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RESEARCH ARTICLE

Importance of Individual Germination Receptor Subunits in the Cooperative Function between GerA and Ynd

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ABSTRACT Germination of *Bacillus* spores is triggered by the binding of specific nutrients to germinant receptors (GRs) located in the spore's inner membrane. The GRs typically consist of A, B, and C subunits, encoded by tricistronic *ger* operons. The *Bacillus licheniformis* genome contains the *gerA* family operons *gerA*, *ynd*, and *gerK*. In contrast to the ABC(D) organization that characterizes *gerA* operons of many *Bacillus* species, *B. licheniformis* genomes contain a pentacistronic *ynd* operon comprising the *yndD*, *yndE*$_3$, *yndE*$_2$, *yndF*$_1$, and *yndE*$_1$ genes encoding A, B, B, C, and B GR subunits, respectively (subscripts indicate paralogs). Here we show that *B. licheniformis* spores can germinate in the absence of the Ynd and GerK GRs, although cooperation between all three GRs is required for optimal germination with amino acids. Spores carrying an incomplete set of Ynd B subunits demonstrated reduced germination efficiencies, while depletion of all three Ynd B subunits restored germination of the spore population to levels only slightly lower than those of wild-type spores at high germinant concentrations. This suggests that the presence of an incomplete set of Ynd B subunits exhibits a dominant negative effect on germination and that the A and C subunits of the Ynd GR are sufficient for the cooperative functionality between Ynd and GerA. In contrast to the B subunits of Ynd, the B subunit of GerA was essential for amino acid-induced germination. This study provides novel insights into the role of individual GR subunits in the cooperative interaction between GRs in triggering spore germination.

IMPORTANCE Spore-forming bacteria are problematic for the food industry, as spores can survive decontamination procedures and subsequently revive in food products, with the risk of food spoilage and foodborne disease. The Ynd and GerA germination receptors (GRs) cooperate in triggering efficient germination of *Bacillus licheniformis* spores when nutrients are present in the surrounding environment. This study shows that the single B subunit of GerA is essential for the cooperative function between Ynd and GerA, while the three B subunits of the Ynd GR are dispensable. The ability of GRs lacking individual subunits to stimulate germination together with other GRs could explain why *ger* operons lacking GR subunit genes are maintained in genomes of spore-forming species.

KEYWORDS *Bacillus licheniformis*, spore germination, germination receptor, endospore, germinant, Ynd, GerK, GerA

When starved for nutrients, many *Bacillus* species differentiate into the endospore (spore) form, which is a metabolically dormant, highly stress-resistant, and nonreproductive differentiation state (1, 2). The spores can stay dormant for long periods and survive environmental stressors that will kill vegetative bacteria (3). When survival conditions improve, the bacteria will initiate their metabolism and rapidly return to vegetative growth through the process of spore germination (3).

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In *Bacillus* species, spore germination can be induced by exposure to biochemical and physical stimuli (3). However, in nature, spore germination is most likely triggered by exposure to nutrient compounds (germinants) such as amino acids, nucleosides, and sugars (3). In *Bacillus* spores, germinants are recognized by the GerA family of germinant receptors (GRs), located in the spore's inner membrane (3). Activation of GRs by their cognate nutrient germinant(s) induces subsequent germination events, including the release of spore's large depot of Ca^{2+}-dipicolinic acid (CaDPA), core rehydration, hydrolysis of the spore's peptidoglycan cortex, and, finally, outgrowth of the vegetative cell (3).

There is a large diversity among GRs with regard to their recognition pattern and responsiveness to germinants, and most *Bacillus* species carry genes encoding several different GerA family GRs (2, 4, 5). Some GRs function alone, while others require cooperation with another GR to trigger spore germination in response to a single germinant compound or a combination of germinant compounds (5). However, it is still largely unknown how the different GRs interact and how the recognition of germinants ultimately leads to spore germination (3). In *Bacillus subtilis*, GRs localize in germinosomes, and the colocalization appears to be essential for efficient spore germination (6, 7). Notably, the germinosomes have always been visualized in defective *cotE* and *gerE* mutants to reduce autofluorescence of the spore's coat layers, but it is not known if this could have influenced the organization of GRs in the spore inner membrane (6, 7).

Molecular, genetic, and biochemical data indicate that GRs are composed of A, B, and C subunits (8). GR genes are usually organized in tricistronic operons, encoding the A, B, and C subunits. However, *gerA* family genes are also found as single genes, in dicistronic operons, or in operons containing more than one gene encoding homologous GR subunits (2, 4). Some *Bacillus* species also encode an additional D subunit, but the function of the D subunit is currently unknown (2, 4). In *Bacillus* species, all A, B, and C subunits appear to be required for a functional GR, but the specific role of each subunit is poorly understood (9). The A and B subunits are predicted to contain 5 and 10 transmembrane domains, respectively, and are therefore almost certainly integral membrane proteins. Recently, the crystal structure of the N-terminal domain of the A subunit of the *Bacillus megaterium* $GerK_3$ GR was resolved (10). Structural analyses revealed that this protein shares structural similarity to substrate binding proteins that serve as receptors for membrane-associated small-molecule transporters and signal transducers. As *B. megaterium* $GerK_3$ is not functional in germination, experimental verification was done in *B. subtilis gerAA* subunits and later in *B. cereus gerIA*. These analyses suggested that the N-terminal domain of GerA is involved directly in the recognition and binding of germinant molecules (10). The B subunit shows homology to proteins of the amino acid/polyamine/organocation (APC) superfamily of single-component membrane transporters (11). However, there is no evidence as to whether the B subunit functions in molecular transport. Evidence based on cross-homologue chimeric constructs and site-directed mutagenesis suggests that the B subunit contains the germinant recognition site, but the identity of the germinant binding site and the mechanism of binding are currently unknown (12–14). In *B. megaterium*, B subunits encoded by different *ger* operons can be used interchangeably in the GerU GR complex, which provides an extended range of recognized germinants (15). In cases where GR operons consist of more than three genes, the B subunit gene is often present in multiple copies. For example, in *B. megaterium* QM B1551, there is an atypical cluster of GR-associated genes, comprised of two B-subunit genes separated by a putative D-subunit gene (16). GR operons possessing multiple B-subunit genes have also been found in the genomes of *Bacillus cereus* E33, *Bacillus halodurans*, *Bacillus cytotoxicus*, *Bacillus licheniformis*, *Clostridium botulinum*, *Clostridium sporogenes*, and *Clostridium acetobutylicum* (4). However, the functional significance of multiple B-subunit genes in the same GR operon remains to be elucidated. The C subunit is a membrane-anchored lipoprotein whose structure has been resolved to a 2.3-Å resolution, but unfortunately, there is still no knowledge on its function (17).

B. licheniformis is widespread in nature and a common food spoilage bacterium

(18–24). It is closely related to *B. subtilis* and has occasionally been associated with disease in humans and abortions in cattle (25–31). *B. licheniformis* carries three *gerA* family operons (*gerA*, *gerK*, and *ynd*), and some strains also carry a monocistronic *yndF2* gene (TRNA_RS32565) (4, 32, 33). In contrast to the ABC(D) organization that characterizes *gerA* family operons in *B. subtilis*, the *B. licheniformis* type strain ATCC 14580/DSM13 possesses a pentacistronic *ynd* operon with the gene organization *yndD* (TRNA_RS32310), $yndE_3$ (TRNA_RS32305), $yndE_2$ (TRNA_RS32300), $yndF_1$ (TRNA_RS32295), and $yndE_1$ (TRNA_RS32290), encoding the GR A, B, B, C, and B subunits, respectively (subscripts indicate paralogs). *B. licheniformis* spores germinate in response to a range of different amino acids as well as glucose, but alanine, cysteine, and valine are the most potent germinants (34). Mutational analyses have shown that GerA, Ynd, and GerK GRs are all functional in nutrient-induced germination of *B. licheniformis* spores (34). GerA appears to function as the primary GR in amino acid-induced germination, but it seems to require intact Ynd and GerK GRs to stimulate efficient germination in response to the above-mentioned germinants (34). A *B. licheniformis* Δ*gerAA-C* mutant (lacking the entire *gerA* operon; the hyphen indicates "from *A* to *C*") showed no detectable germination with 100 mM alanine and valine and only very weak germination with 100 mM cysteine, which suggests that Ynd and GerK do not function alone in triggering efficient germination with the tested amino acids (34). A Δ*gerAA* Δ*gerKA-C* mutant, which still expresses the intact Ynd GR and the B and C subunits of the GerA GR, showed detectable germination with 100 mM alanine and cysteine (34). The same study showed that deletion of *yndD* in the Δ*gerAA* Δ*gerKA-C* mutant background resulted in spores that showed no germination. No functional interdependence between the Ynd and GerK GRs has been found, but similar to GerK in *B. subtilis* and *B. megaterium*, GerK is required for D-glucose-induced germination of *B. licheniformis* spores. However, D-glucose functions as only a weak germinant for *B. licheniformis* spores (34).

The purpose of this study is to further examine the cooperative function between GerA and Ynd. In particular, by using mutational analyses, this study addresses the functional role of the three paralogous B-subunit genes in the *ynd* operon in the interplay between the Ynd and GerA GRs. The functional importance of the orphan $yndF_2$ gene, encoding a C subunit of GerA family GRs, was also investigated.

RESULTS

GerA functions in spore germination in the absence of other germination receptors, and the GerA B subunit is essential for its functionality. It has previously been shown that Ynd requires GerA to function in *B. licheniformis* spore germination. However, it is not known whether GerA can function individually or is dependent on Ynd or GerK to be functional. To examine this further, a *B. licheniformis* mutant strain lacking both the entire *ynd* and *gerK* operons (Δ$yndDE_3E_2F_1E_1$ Δ*gerKACB*) was constructed. Spores of this mutant (strain NVH-1412), which expresses only a functional GerA GR, still germinated with L-alanine, L-cysteine, and L-valine although with a much lower efficiency than for spores of the wild-type background strain (Table 1). To test the importance of the B subunit of GerA in the functionality of the GR, a mutant strain carrying a *gerAB* in-frame deletion was constructed (NVH-1389). Spores of this mutant showed no detectable germination response after 2 h of germinant exposure (Table 1), which indicates that the B subunit of the GerA GR is essential for *B. licheniformis* spore germination with L-alanine, L-cysteine, and L-valine.

The *ynd* operon is highly conserved among *B. licheniformis* strains. *B. licheniformis* and *Bacillus paralicheniformis* genomes were explored for the presence of the *ynd* operon using the complete *yndD* nucleotide sequence from *B. licheniformis* ATCC 14580/DSM13 as the seed sequence. Pentacistronic *ynd* operons were found in all 25 *B. licheniformis* strains investigated. However, five genomes carried *ynd* operons with premature stop codons in the *yndE* genes (4/5) or in the $yndF_1$ gene (1/5) (results not shown). These genomes were excluded from the phylogenetic analysis. All remaining 17 *B. licheniformis* and 3 *B. paralicheniformis* (9945a, BL09, and 12759) strains possessed

TABLE 1 Germination properties of *B. licheniformis* wild-type and mutant strains[a]

Strain	Genotype	Protein(s) present	Mean no. of spores by microscopic count ± SD[b]			Mean G_{max} ± SD[c]		
			L-Ala	L-Cys	L-Val	L-Ala	L-Cys	L-Val
WT[d]		Ynd, GerA, GerK	96 ± 1	97 ± 1	78 ± 13	−1.5 ± 0.3	−1.9 ± 0.4	−0.7 ± 0.2
NVH-1387	Δ*yndDE$_3$E$_2$F$_1$E$_1$*	GerA, GerK	47 ± 7	36 ± 10	52 ± 5	−0.4 ± 0.2	−0.1 ± 0.0	−0.4 ± .0.1
NVH-1412	Δ*yndDE$_3$E$_2$F$_1$E$_1$* Δ*gerKACB*	GerA	46 ± 2	39 ± 0	47 ± 2	−0.3 ± 0.1	−0.2 ± 0.0	−0.3 ± .0.0
NVH-1389	Δ*gerAB*	Ynd, GerK, GerAA, GerAC	2 ± 2	4 ± 4	2 ± 1	−0.1 ± 0.0	−0.1 ± 0.0	−0.1 ± .0.0
NVH-1369	Δ*yndE$_3$*	YndDE$_2$F$_1$E$_1$, GerA, GerK	58 ± 6	48 ± 8	57 ± 1	−0.4 ± 0.0	−0.4 ± 0.0	−0.4 ± .0.0
NVH-1378	Δ*yndE$_3$E$_2$*	YnDF$_1$E$_1$, GerA, GerK	50 ± 4	41 ± 3	53 ± 6	−0.5 ± 0.1	−0.4 ± 0.2	−0.5 ± .0.2
NVH-1405	Δ*yndE$_2$E$_3$E$_1$*	YndDF$_1$, GerA, GerK	86 ± 5	76 ± 7	85 ± 5	−0.9 ± 0.3	−0.6 ± 0.1	−0.8 ± .0.2
NVH-1371	Δ*yndF$_2$*	Ynd, GerA, GerK	95 ± 4	98 ± 1	70 ± 12	−1.5 ± 0.4	−1.8 ± 0.2	−0.5 ± .0.2
NVH-1404	NVH-1387 Δ*gerKA-C*	GerA, GerKB	47 ± 5	42 ± 8	55 ± 3	−0.4 ± 0.1	−0.3 ± 0.1	−0.4 ± .0.1
Complementations								
cis								
NVH-1421	NVH-1369::*yndE$_3$*		95 ± 2	97 ± 1	75 ± 24	−1.3 ± 0.2	−1.3 ± 0.3	−0.7 ± .0.1
NVH-1416	NVH-1405::*yndE$_3$E$_2$*		85 ± 6	81 ± 4	80 ± 5	−0.7 ± 0.0	−0.6 ± 0.0	−0.6 ± 0.1
NVH-1417	NVH-1405::*yndE$_3$E$_2$E$_1$*		95 ± 4	94 ± 4	89 ± 6	−1.4 ± 0.3	−1.0 ± 0.0	−0.9 ± .0.0
NVH-1413	NVH-1404::*yndDE$_2$F$_1$*		47 ± 5	48 ± 5	37 ± 3	−0.5 ± 0.0	−0.4 ± 0.0	−0.3 ± .0.0
NVH-1427	NVH-1412::*yndDF$_1$*		69 ± 6	60 ± 3	71 ± 4	−0.4 ± 0.0	−0.3 ± 0.0	−0.4 ± 0.0
trans								
NVH-1474	NVH-1412/pHT315		28 ± 5	14 ± 6	27 ± 1	−0.2 ± 0.0	−0.1 ± 0.0	−0.2 ± .0.0
NVH-1438	NVH-1412/pHT315-*yndDF$_1$*		49 ± 9	36 ± 11	55 ± 10	−0.4 ± 0.1	−0.2 ± 0.1	−0.4 ± .0.1
NVH-1473	NVH-1412/pHT315-*yndD*		24 ± 3	14 ± 4	27 ± 4	−0.2 ± 0.0	−0.1 ± 0.0	−0.2 ± .0.0

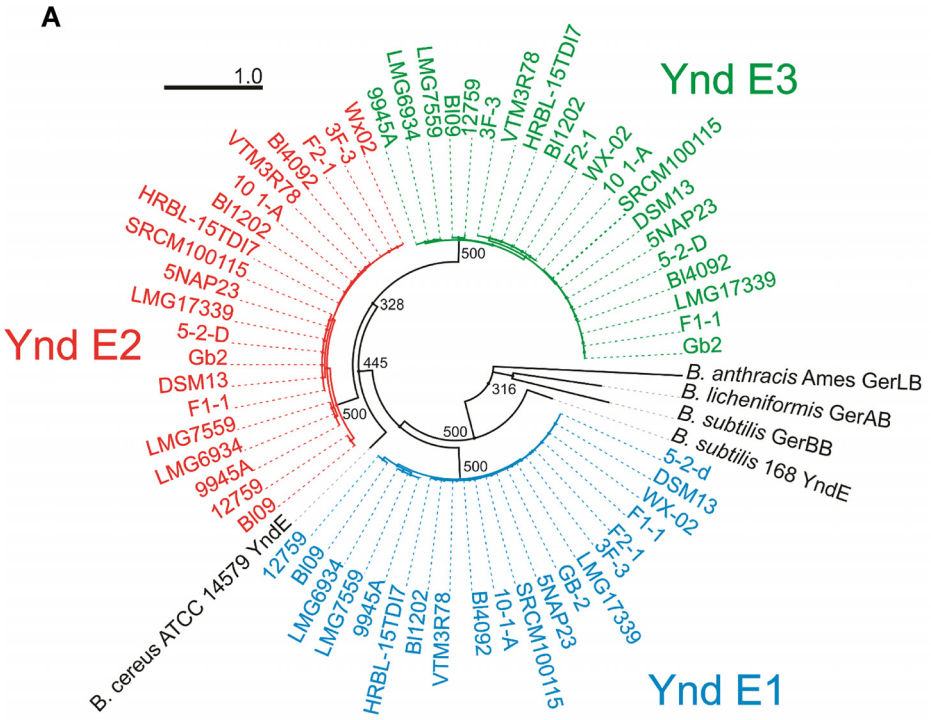
[a]All data are presented as means from three biological replications.
[b]The percentages of germinated (phase-dark) spores were determined after 120 min of exposure to 100 mM germinant compounds.
[c]G_{max} is the maximum rate of germination (ΔOD_{600} units per minute).
[d]The wild-type (WT) strain shows 1% ± 1% germination with just buffer (no germinants added), as determined by microscopic counts.

atypical pentacistronic *ynd* operons with a *yndD*, *yndE$_3$*, *yndE$_2$*, *yndF$_1$*, and *yndE$_1$* gene organization similar to the one found in the *B. licheniformis* type strain ATCC 14580/DSM13. A maximum likelihood phylogram based on alignment of the deduced amino acid sequences of *yndE* genes from the 17 *B. licheniformis* and 3 *B. paralicheniformis* strains revealed a monophyletic clade with three distinct branches corresponding to YndE$_1$, YndE$_2$, and YndE$_3$ (Fig. 1A).

The amino acid sequences of the three *B. licheniformis* YndE subunits are resolved as monophyletic groups, each with 100% bootstrap support. YndE$_2$ and YndE$_3$ appear to be more closely related to each other than to YndE$_1$, as determined by phylogenetic analyses of the *yndE* genes of the *B. licheniformis* strains (Fig. 1A). Accordingly, alignments of *B. licheniformis* DSM13/ATCC 14580 amino acid sequences showed that YndE$_3$ and YndE$_2$ shared more identity (64%) than YndE$_1$ and YndE$_2$ or YndE$_3$ (52% and 54%, respectively) (Fig. 1B; see also Table S2 in the supplemental material).

All *ynd* genes are expressed during sporulation. Expression analysis of *B. licheniformis* grown in sporulation medium revealed that the *yndE$_3$* and *yndE$_2$* genes were expressed at approximately the same level as *yndD* and *yndF$_1$* during sporulation of the type strain derivative MW3 (Fig. 2). The impression during the analysis was that *yndE$_1$* was transcribed at a level slightly higher than those of the other genes of the operon; however, it was statistically significant only by pairwise comparisons using two-tailed paired Student *t* tests ($P < 0.05$) at 21 h. The transcription level of the individual *ynd* genes increased approximately five times from 5 h (late exponential growth) to 8 h (early stationary growth) after inoculation and was between 0.01 and 0.1 times the expression of *rpoB* at 21 h, when there were about 50% spores in the culture, as observed by phase-contrast microscopy (Fig. 2).

The Ynd AC subunits are functional in germination in the absence of the Ynd B subunits. To examine the contribution of the Ynd B subunits to spore germination, spores of a mutant carrying in-frame deletions of all three *yndE* genes (NVH-1405) were tested for germination with L-alanine, L-cysteine, and L-valine. Despite the absence of all B subunits, Δ*yndE$_3$E$_2$E$_1$* mutant spores germinated in a manner similar to that of



B

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E1 -MIRSEDKITTSQAIVVLVSYIMAAGILTLPRTAVEEAGTPDVWISVLLGGLVTTAAGVV 59
E2 MMVGQQDKITVSQTSIVITNFLLGTGILTLPRTSGEAVKAPDVWLSVAFGGLVAILAGFI 60
E3 MMVRHEERITTSQTAVLITNFLLGTGILTLPRTSVDEVGTPDVWITLVLGGLVALTAAVI 60

E1 MTKLSQHYFGQTFFQYGGDIVGKWLSIVISLLFIEHFVAIAAFEARILAEVITFFLLQQT 119
E2 MVKLSMEYFDKTLYQYSRDLIGKWPGGLFTLLLICYFLATSGFQVRSVTEVMGFYLLEET 120
E3 MVKLNHQYFDKTFYFYSQDIIGKWGGSLISLFFACYLLAHSGFQVRSMLEIIRFFLLEGT 120

E1 PIWAIVMSMMWIGFYSIKDGMGSLARLFEIIFPITILFFLTVVFMSFGIFELDNLRPVLG 179
E2 PIWATAMVFLWSGIYLLTGGINPLARLYEIIFPITVLIFLIVIFMSASIFDIDNLRPVLG 180
E3 PRWAVAMVFLWVGLYLIVGGLSSIARLFQIVLPITFILFLLVVFMSIGIFDVDNLRPVLG 180

E1 KGIMPALKGIKATTLSFISAEVMVFLLAFMKKPKDGIKVVVMGIGIAMLFYLTTIVFVIG 239
E2 KGIKPVLEGVKITSFAYTGIEIILIIVPFMRKPEQALKATLMGISFPIVFYMITVIMVIG 240
E3 EGIGPVLKGIKVTALSFSGIEIILILMPFMNQPRKAIHTIMIGIAIPLLFYMITVVMVIG 240

E1 GLSVDGVTAMIWPALDLIRSFEMPGLIFERFESLLLVIWIMQLFATFSVSYYAASLGLSQ 299
E2 ALAIEGVTSRTWPTIDLMRSFEIPGLIFERFESLLLVIWIMQMFSSFTISYFAAALGLSQ 300
E3 ALSIDGVVSRTWPTIDLMRSFEIPGLIFERFESLLLVIWIMQLFCTFAIAFYGSALGLAQ 300

E1 IFKKKSRPIMFGLLPVMYLLAMTPKNIDDVFSLGDMIGYIAAVLFGIVPIPLLLVSKMRK 359
E2 LLKKRMEPILFSLLPVIYLIAMAPKNINQLFQMGDLIGNSAIYLVGLIPLMLLVISKVRR 360
E3 LFKTNIRPMMFVLLPVIYLIAMIPKNINAMFAFGDFIGNSGVYLGGLTPLVLLVISKIRR 360

E1 KKAVQS 365
E2 KKFEKN 366
E3 RKQGIS 366
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FIG 1 (A) Maximum likelihood phylogram for $YndE_1$, $YndE_2$, and $YndE_3$ of 20 strains of *B. licheniformis* as well as of *B. cereus* and *B. subtilis*. Bootstrap support values above 50% are indicated at the nodes; however, no bootstrap support values for nodes within the clades for the three *B. licheniformis* Ynd genes are given. GerLB, GerAB, and GerBB from *B. anthracis*, *B. licheniformis*, and *B. subtilis*, respectively, were used as the outgroups. (B) Amino acid sequence alignment of the Ynd B subunits encoded by the $yndE_3$, $yndE_2$, and $yndE_1$ genes of *B. licheniformis* DSM13/ATCC 14580.

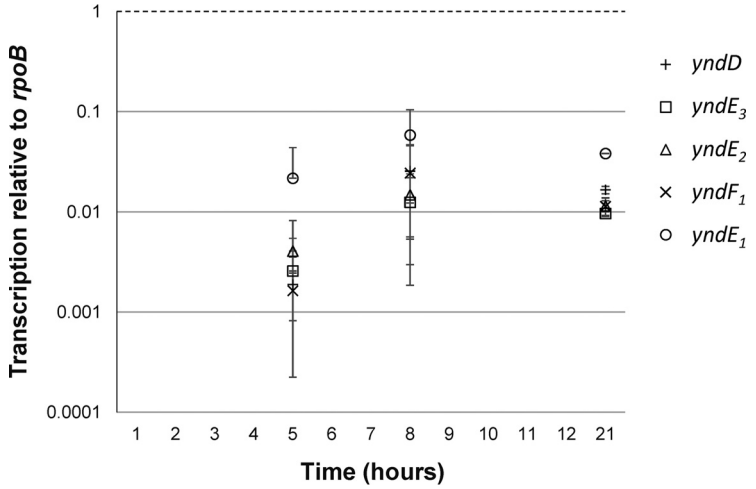

FIG 2 Transcription levels of *yndD*, *yndE*$_3$, *yndE*$_2$, *yndF*$_1$, and *yndE*$_1$ relative to *rpoB* determined by RT-qPCR during 21 h of growth of *B. licheniformis* MW3. At 21 h, there were about 50% spores in the cultures, as observed by phase-contrast microscopy. Whiskers represent standard deviations of the means based on data from three independent experiments.

wild-type spores with 100 mM germinants, and their germination rate was only slightly lower than that of wild-type spores (Fig. 3 and Table 1). Wild-type and Δ*yndE*$_3$*E*$_2$*E*$_1$ mutant spores demonstrated similar germination levels when lower concentrations of L-alanine and L-cysteine were used, but the mutant spores showed slightly improved germination compared to wild-type spores with L-valine at all concentrations tested (Table 2).

It was previously shown that compounds other than L-alanine, L-cysteine, and L-valine functions as moderate (L-serine, L-isoleucine, and L-aspartic acid) and weak (D-glucose, L-methionine, and L-lysine) germinants for *B. licheniformis* MW3 spores (34). When Δ*yndE*$_3$*E*$_2$*E*$_1$ mutant spores were tested for germination with 100 mM these compounds, they demonstrated a slightly improved germination response toward L-methionine compared to wild-type spores (Table 3); however, the improvement was not statistically significant. The similar germination efficiencies of spores of the wild type and of the Δ*yndE*$_3$*E*$_2$*E*$_1$ mutant after 2 h of exposure to germinants (Table 2) suggest that the Ynd B subunits do not play an important role in germination with these compounds. When Δ*yndE*$_3$*E*$_2$*E*$_1$ mutant spores were *cis* complemented with *yndE*$_3$*E*$_2$*E*$_1$ (NVH-1417), the germination responses with L-alanine, L-cysteine, and L-valine were similar to those of wild-type spores (Table 1).

The more efficient germination observed for Δ*yndE*$_3$*E*$_2$*E*$_1$ spores than for Δ*yndDE*$_3$*E*$_2$*F*$_1$*E*$_1$ spores suggests that the A and C subunits of Ynd are sufficient for an effective germination response. Deletion of the whole *gerK* operon (Δ*gerKACB*) in the

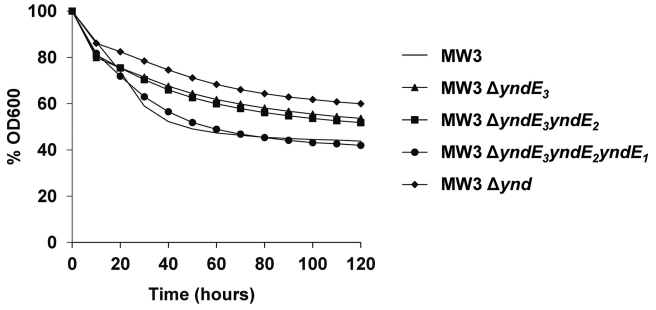

FIG 3 Spore germination of *yndE*$_1$, *yndE*$_1$*E*$_2$, and *yndE*$_3$*E*$_2$*E*$_1$ deletion mutants and of the mutant lacking the entire *ynd* operon. All mutants were derived from the MW3 strain. Germination was measured by the decrease in the OD_{600} over a period of 120 min after the addition of the germinant.

TABLE 2 Germination of wild-type and Δ*yndE*$_2$*E*$_3$*E*$_1$ mutant spores at different concentrations of germinants

Amino acid concn (mM)	Mean germination rate (%) ± SD[a]					
	WT			Δ*yndE*$_2$*E*$_3$*E*$_1$ mutant		
	L-Ala	L-Cys	L-Val	L-Ala	L-Cys	L-Val
100	96 ± 1	97 ± 1	79 ± 10	88 ± 2	84 ± 6	88 ± 4
10	73 ± 16	80 ± 16	35 ± 12	70 ± 7	69 ± 2	65 ± 3
5	56 ± 20	51 ± 22	19 ± 9	60 ± 7	40 ± 1	49 ± 1
1	44 ± 21	3 ± 1	3 ± 2	54 ± 4	4 ± 2	21 ± 4
0.5	30 ± 14	2 ± 1	1 ± 0	43 ± 7	2 ± 1	6 ± 2

[a]All data are presented as means from three biological replications. The percentages of germinated (phase-dark) spores were determined by phase-contrast microscopy after 120 min of exposure to decreasing concentrations of germinants.

Δ*yndDE*$_3$*E*$_2$*F*$_1$*E*$_1$ background (NVH-1412) did not alter the germination properties compared to the background strain (Table 1), which indicates that GerK does not contribute significantly to the observed germination. To test whether both the A and C subunits of Ynd are required for the cooperation between GerA and Ynd, attempts were made to construct a *yndE*$_3$*E*$_2$*F*$_1$*E*$_1$-null mutant, but despite several attempts, we were not able to construct this mutant. Several attempts to make *cis* complementations with *yndD* and *yndDF*$_1$ in the Δ*yndDE*$_3$*E*$_2$*F*$_1$*E*$_1$ mutant also failed for an unknown reason. However, *trans* complementation of the Δ*yndDE*$_3$*E*$_2$*F*$_1$*E*$_1$ Δ*gerKACB* mutant (NVH-1412) with *yndD* and *yndDF*$_1$ gave some new insight. NVH-1412 spores carrying the empty pHT315 vector showed a reduced maximum germination rate (G_{max}), and the number of germinated spores as measured by microscopic counts was reduced about 50% compared to spores of the background NVH-1412 strain, indicating that the plasmid had a negative effect on germination (Table 1). Accordingly, in order to avoid bias due to the presence or absence of the plasmid, germination properties of the complemented spores were compared to those of spores of NVH-1412 carrying the empty pHT315 vector. *trans* complementation of NVH-1412 with *yndD* (NVH-1473, encoding the A subunit) resulted in spores that showed the same germinability as NVH-1412 spores carrying the empty vector, while complementation with *yndDF*$_1$ (NVH-1438, encoding the A and C subunits) resulted in spores showing germination approximately twice as effective as that of NVH-1412 spores with the empty pHT315 vector (Table 1). This indicates that at least the C subunit of Ynd is needed to trigger efficient germination in cooperation with GerA and that the A subunit alone is not sufficient for the cooperative functionality between Ynd and GerA. *cis* complementation of NVH-1412 with *yndDF*$_1$ also demonstrated an increased germination response compared to that of NVH-1412 (Table 1).

The individual Ynd B subunits contribute differently to spore germination. The separate phylogenetic clustering of YndE$_1$, YndE$_2$, and YndE$_3$ (Fig. 1A) suggests that they may perform different functions. To explore the function of individual B subunits, mutants lacking one or several Ynd B subunits were tested for germination with 100

TABLE 3 Germination of wild-type and Δ*yndE*$_2$*E*$_3$*E*$_1$ mutant spores in response to other amino acids

Germinant (100 mM)	Mean germination rate (%) ± SD[a]	
	WT	Δ*yndE*$_3$*E*$_2$*E*$_1$ mutant
L-Methionine	16 ± 7	48 ± 22
D-Glucose	2 ± 1	9 ± 7
L-Lysine	1 ± 0	3 ± 2
L-Serine	48 ± 28	33 ± 22
L-Isoleucine	53 ± 10	76 ± 22

[a]All data are presented as means from three biological replications. The percentages of germinated (phase-dark) spores were determined by phase-contrast microscopy after 120 min of exposure to 100 mM germinant compounds.

mM L-alanine, L-cysteine, and L-valine. Spores of the Δ*yndE*$_3$ mutant showed germination that was reduced to levels comparable to those of Δ*yndDE*$_3$*E*$_2$*F*$_1$*E*$_1$ mutant spores lacking the whole *ynd* operon (Table 1 and Fig. 3). Wild-type levels of spore germination were restored when the Δ*yndE*$_3$ mutant was *cis* complemented with the *yndE*$_3$ gene (NVH-1421) (Table 1). No further reduction in total germination compared to the Δ*yndE*$_3$ mutant spores was observed when both *yndE*$_2$ and *yndE*$_3$ were deleted simultaneously (NVH-1378) (Table 1). Next, the mutant where the entire *ynd* operon was deleted was *cis* complemented with *yndDE*$_2$*F*$_1$. Spores of the resulting NVH-1413 strain, which express YndDE$_2$F$_1$, showed severely reduced germinability (Table 1). Together, these results suggest that the YndE$_3$ subunit plays an important role in germination with the tested germinants, while YndE$_2$ plays a minor role, if any. For an unknown reason, we were not able to construct a Δ*yndE*$_1$ mutant. Therefore, to investigate the role of YndE$_1$ in germination, a Δ*yndE*$_3$*E*$_2$*E*$_1$ mutant (NVH-1405) was *cis* complemented with Δ*yndE*$_3$*E*$_2$. Spores of the resulting strain (NVH-1416) (Table 1), which lack only the YndE$_1$ subunit of the Ynd GR, demonstrated slightly reduced total germination after 2 h of germinant exposure and a 2-fold-reduced G_{max} compared to wild-type spores. These results suggest that YndE$_1$ also contributes to germination with L-alanine, L-cysteine, and L-valine but is less important than YndE$_3$. Notably, spores of strain NVH-1405, which lacks all three YndE subunits, germinated markedly more efficiently than did spores lacking either or both of the YndE$_2$ and YndE$_3$ subunits.

Analysis of YndF$_2$. The orphan *yndF*$_2$ gene was present in 12 of the 20 *B. licheniformis* genomes analyzed in this study. The deduced sequence of YndF$_2$ from ATCC 14580/DSM13 is 184 amino acids long and shows 60% identity and 79% similarity to the C-terminal region of YndF$_1$ (Table S2). However, in the majority of the 12 investigated genomes where YndF$_2$ is present, it is 401 amino acids long, which strongly suggests that YndF$_2$ from ATCC 14580/DSM13 is truncated at the N-terminal end. We were unable to identify a promoter upstream of *yndF*$_2$ in strain ATCC 14580/DSM13; however, analysis of its expression by reverse transcriptase quantitative PCR (RT-qPCR) revealed that *yndF*$_2$ is transcribed at approximately the same level as the *yndE* genes (data not shown). Therefore, to evaluate its potential role in spore germination, we constructed a mutant strain carrying an in-frame deletion of *yndF*$_2$ (NVH-1371). Spores of the resulting mutant demonstrated germination efficiencies similar to those of wild-type spores (Table 1), suggesting that YndF$_2$ is not important for germination with the tested amino acids.

DISCUSSION

The main objective of the present study was to increase the understanding of the functional interaction occurring between cooperating GRs during nutrient-triggered spore germination, using *B. licheniformis* GerA and Ynd as models. This work also attempts to characterize the role of the paralogous B subunits encoded by the *yndE*$_3$, *yndE*$_2$, and *yndE*$_1$ genes in *B. licheniformis* spore germination.

B. licheniformis is so far the only species known to contain a functional Ynd GR. The *ynd* operon is highly conserved among the *B. licheniformis* and *B. paralicheniformis* strains investigated in this study. In contrast, the selection pressure for maintaining this GR seems to be much lower among *B. subtilis* strains (35). Phylogenetic examination of *yndE* genes from 17 *B. licheniformis* and 3 *B. paralicheniformis* strains showed that YndE$_1$, YndE$_2$, and YndE$_3$ form three separate phylogenetic clusters, suggesting that individual B subunits play different functional roles. Interestingly, the *B. cereus yndE* gene forms a clade with *B. licheniformis yndE*$_2$ and *yndE*$_3$. Two scenarios can explain the observed phylogeny. A putative horizontally transferred copy of the *B. cereus yndE* gene might have been acquired and subsequently duplicated into *yndE*$_2$ and *yndE*$_3$ in *B. licheniformis*. Alternatively, duplication of an ancestral *B. licheniformis yndE* gene resulted in *yndE*$_1$ and an ancestral paralogue that subsequently duplicated into *yndE*$_2$ and *yndE*$_3$. In this scenario, *B. cereus* might have received the ancestral paralogue from *B. licheniformis*. *B. subtilis* YndE also has an uncertain placement due to low support values

but is either sister to *B. licheniformis* $YndE_3$ or $YndE_2$ and *B. cereus* YndE or is sister to *B. licheniformis* $YndE_1$.

Transcriptional analyses revealed that the *yndE*$_3$, *yndE*$_2$, and *yndE*$_1$ genes are expressed during sporulation. The absence of *yndE*$_3$ or both *yndE*$_3$ and *yndE*$_2$ resulted in weaker germination responses with L-alanine, L-cysteine, and L-valine than for wild-type spores, indicating that the Ynd GR could not function optimally in the absence of $YndE_3$ in triggering germination in cooperation with GerA. Deletion of *yndE*$_1$ also seemed to have a negative effect on germination although not as pronounced as what was seen for spores of mutants lacking $YndE_3$. Remarkably, simultaneous deletion of the *yndE*$_1$, *yndE*$_2$, and *yndE*$_3$ genes restored the germination rate and total germination of the spore population to levels only slightly lower than those of wild-type spores. While spores carrying an incomplete set of Ynd B subunits showed reduced germination efficiencies, spores lacking all three Ynd B subunits germinated almost as efficiently as wild-type spores (Tables 1 and 2). This indicates that the presence of an incomplete set of Ynd B subunits exhibits a dominant negative effect on germination properties. Since all *B. licheniformis* and *B. paralicheniformis* genomes investigated in this study encode the GerD protein, which facilitates germinosome formation in *B. subtilis* (at least in defective *cotE* and *gerE* mutants), it is likely that GerA, Ynd, and GerK are colocalized in germinosomes. However, there is very little knowledge on the heteromeric organization and potential clustering of GRs in the spore's inner membrane, and therefore, the interpretation of the physiological basis for our results remains speculative. Assuming that GerA and Ynd form a heteromeric complex in the spore's inner membrane, the absence of one or more of the encoded Ynd B subunits may obstruct the organization of the GR complex, resulting in a dominant negative effect on the cooperative function between Ynd and GerA (Tables 1 and 2). However, when all Ynd B subunits are absent, the remaining GerAB subunit alone may support the cooperative function between Ynd and GerA. The B subunit of the GerA GR was in contrast to the B subunits of the Ynd GR indispensable for *B. licheniformis* spore germination with L-alanine, L-cysteine, and L-valine, which indicates that the absence of GerAB cannot be functionally complemented with B subunits in the Ynd or GerK GR. Similarly, some amino acid substitutions in *B. subtilis gerAB* have been shown to result in a loss of spore germination with L-alanine and an apparent loss of the GerA GR as judged by the loss of the GerAC protein from crude spore extracts (36).

B. licheniformis GerA is necessary and sufficient for germination with all tested germinant compounds, and the presence of an intact Ynd GR is important for wild-type levels of germination with L-alanine, L-cysteine, and L-valine, especially at low germinant concentrations (34, 37). Previously, we have shown that a Δ*gerAA* Δ*gerKA-C* mutant, which still expressed the intact Ynd GR and the B and C subunits of the GerA GR, showed detectable germination with 100 mM alanine and cysteine (34). The same study revealed that deletion of *yndD* in the Δ*gerAA* Δ*gerKA-C* mutant background resulted in spores that showed no germination at all. Together, these results indicate that the Ynd GR can complement the absence of the GerA A subunit to some extent and that the functions of these GRs are tightly interconnected. The interdependence between the GerA and the Ynd GRs is important for a robust germination response and could be a major determinant for maintaining the *ynd* operon in *B. licheniformis* genomes. The B subunit of GRs has been suggested to contain the germinant binding site, as amino acid substitutions in *B. megaterium* GerVB changed the germinant recognition specificity (12, 38). However, the three B subunits in the *B. licheniformis* Ynd GR do not appear to play a major role in germination with the tested nutrients, as Δ*yndE*$_3$*E*$_2$*E*$_1$ mutant spores, which lack all Ynd B subunits, did not differ from wild-type spores in the repertoire of nutrients recognized as germinants (Tables 2 and 3). It is likely, however, that the structure of the Ynd GR in *B. licheniformis* reflects an ecological specialization, and it could have a more pronounced function in complex natural soil environments where the spores can be exposed a wide range of different nutrient compounds.

Mutant spores containing the GerA GR and the A and C subunits of the Ynd GR (Δ*yndE*$_3$*E*$_2$*E*$_1$) demonstrated near-wild-type efficiencies of spore germination with

L-alanine, L-cysteine, and L-valine, suggesting that the A and C subunits encoded by the *ynd* operon are sufficient for the functional cooperation between Ynd and GerA. Further study of spores lacking the entire *ynd* operon (Δ*yndDE*$_3$*E*$_2$*F*$_1$*E*$_1$) but complemented with the *yndD* gene showed that they germinated with the same efficiency as Δ*yndDE*$_3$*E*$_2$*F*$_1$*E*$_1$ spores, which lack the whole Ynd GR, suggesting that at least the C subunit is essential for functional cooperation between Ynd and GerA. Dicistronic operons encoding A and C, A and B, and B and C subunits and monocistronic operons encoding single A, B, and C subunits are found in many *Bacillales* and *Clostridiales* genomes (2). Whether a GR encoded by such "atypical" *gerA* family operons can be capable of functioning alone or whether they function only in cooperation with other GRs remains to be clarified. However, the ability of the A and C subunits of the *B. licheniformis* Ynd GR to mediate a functional interaction with GerA suggests that GRs containing only the A and C subunits might also be functional in other species, at least in cooperation with other GRs. A situation where a GR does not require all subunits to function in triggering spore germination has been described in *C. perfringens*, which contains a bicistronic *gerK* operon encoding an A subunit and a C subunit, which can function in the absence of a B subunit (39, 40). Interestingly, the C subunit was found to be essential for *C. perfringens* spore germination, whereas the A subunit was dispensable (41). Additional studies are needed to address whether this is also the case for the A and C subunits of the *B. licheniformis* Ynd GR.

The YndF$_2$ subunit was found to be dispensable for *B. licheniformis* spore germination with all tested germinant compounds. However, *yndF*$_2$ appears to be truncated in strain ATCC 14580/DSM13, while it is present as a full-length (1,200-bp) gene in most other *B. licheniformis* genomes. It is therefore possible that it plays a functional role in other *B. licheniformis* strains, but this has yet to be determined.

The methods to assess germination used in this work determine the average germination kinetics and total level of germination for the entire spore population. These methods are useful for studying the germination behaviors of a range of different GR mutants with different germinant compounds, but since spore populations generally are very heterogeneous, they do not provide information about the behavior of individual spores or differentiate between different germination phases. However, single-spore analyses such as live imaging, laser tweezers Raman spectroscopy, and/or flow cytometry analysis are more laborious but will provide more detailed information about the behavior of individual spores and differentiates better between the time to start germination and the time of the germination process itself (42–44).

MATERIALS AND METHODS

Strains. The *B. licheniformis* strains used in this study are listed in Table 4. Strain MW3 carries deletions in the *hsdR* loci encoding two type I restriction-modification systems and is therefore a more readily transformable derivative of the *B. licheniformis* type strain DSM13 (45).

Bioinformatic analyses. The *ynd* gene sequences of *B. licheniformis* type strain DSM13/ATCC 14580, *B. subtilis* strain 168, *B. cereus* strain ATCC 14579, and the panel of 20 *B. licheniformis* and *B. paralicheniformis* strains were acquired from the NCBI database, including both complete and draft assembled genomes (www.ncbi.nlm.nih.gov/). The *ynd* genes were identified using nBLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), using the *ynd* genes from strain DSM13/ATCC 14580 as query sequences. To investigate the evolutionary relationships between *yndE* genes, we performed a phylogenetic analysis. The deduced amino acid sequences of the three *yndE* genes from each of the 20 strains of *B. licheniformis* and *B. paralicheniformis* were aligned using ClustalX, followed by manual inspection in BioEdit (46). We also included the deduced amino acid sequences of the *yndE* genes of *B. subtilis* and *B. cereus*. As outgroups, we used GerLB from *B. anthracis*, GerAB from *B. licheniformis*, and GerBB from *B. subtilis*. Based on the Akaike information criterion calculated in SMS (47), we estimated maximum likelihood phylogenies from the data under the LG+G+F substitution model using PhyML (48) with 500 bootstrap replicates.

Analyses of relative gene expression using RT-qPCR. The relative expression levels of *yndE*$_1$, *yndE*$_2$, *yndE*$_3$, *yndF*$_2$, and *yndD* relative to *rpoB* were determined using reverse transcriptase quantitative PCR (RT-qPCR). Sporulation for RNA extraction, cDNA synthesis, and RT-qPCR analysis was performed as described previously (34, 49). RT-qPCR was performed in triplicates on three biological replicates, and the results were analyzed as described previously (34, 49). All primers used for RT-qPCR analyses are listed in Table S1 in the supplemental material.

Construction of deletion mutants. In-frame deletion mutants were constructed by replacing the target gene(s) with the nucleotide sequence 5′-ATGTAR-3′ (where R is A or G) using a markerless gene replacement method (50), as described previously (37). This method results in an in-frame deletion of the

TABLE 4 Strains used in this study

Strain	Description or genotype	Reference(s)
ATCC 14580/DSM13	Type strain	32, 33
MW3	DSM13 Δ*hsdR1* Δ*hsdR2*	45
NVH-1387	Δ*yndDE*$_3$*E*$_2$*F*$_1$*E*$_1$	This study
NVH-1412	Δ*ynd* Δ*gerKACB*	This study
NVH-1389	Δ*gerAB*	This study
NVH-1369	Δ*yndE*$_3$	This study
NVH-1378	Δ*yndE*$_3$*E*$_2$	This study
NVH-1405	Δ*yndE*$_2$*E*$_3$*E*$_1$	This study
NVH-1371	Δ*yndF*$_2$	This study
NVH-1404	NVH-1387 Δ*gerKA-C*	This study
NVH-1421	NVH-1369::*yndE*$_3$	This study
NVH-1416	NVH-1405::*yndE*$_3$*E*$_2$	This study
NVH-1417	NVH-1405::*yndE*$_3$*E*$_2$*E*$_1$	This study
NVH-1413	NVH-1404::*yndDE*$_2$*F*$_1$	This study
NVH-1427	NVH-1412::*yndDF*$_1$	This study
NVH-1474	NVH-1412/pHT315	This study
NVH-1438	NVH-1412/pHT315-*yndDF*$_1$	This study
NVH-1473	NVH-1412/pHT315-*yndD*	This study

target gene and ensures that the up- and downstream flanking sequences, including the promoter region, are intact. Briefly, the deletion mutants were constructed by amplifying an ~500-bp segment upstream of the target gene, using primers A and B, and an ~500-bp segment downstream of the target gene, using primers C and D. Primers B and C contain a sequence overlap enabling fusion of the AB and CD PCR products by sequence- and ligation-independent cloning (SLIC)-PCR. The resulting AD fragment contains the upstream and downstream sequences of the target gene. The AD fragment was then cloned into a thermosensitive shuttle vector, pMAD (51), containing an I-SceI restriction site (52). The resulting plasmid, pMAD-I-SceI, carrying the gene deletion construct was then transformed into electrocompetent *B. licheniformis* cells (53). Here the whole plasmid construct was expected to integrate into the chromosome by a single crossover. The plasmid pBKJ233, encoding the I-SceI enzyme, was then introduced by electroporation. The I-SceI restriction enzyme makes a double-stranded cut at its recognition site, which allows for recombinational repair, resulting in a second crossover where the target gene is deleted. Deletion of the target gene was confirmed by PCR and sequencing (GATC Biotech). All primers used for construction of the mutant strains are listed in Table S2. All PCR products used for making the deletion mutants were produced using Phusion high-fidelity DNA polymerase (Finnzymes, Finland) according to the manufacturer's instructions. All PCRs were performed using an Eppendorf Mastercycler ep-Gradient S instrument.

Complementation tests. The complementing constructs used for *cis* complementation, comprising the *ynd* promoter region (570 bp) followed by the complementing gene(s), were cloned into pMAD-NotI. The NotI site was introduced into the EcoRI site of pMAD by SLIC-PCR, comprising an upstream part (404 bp) and a downstream part (252 bp) of *B. licheniformis amyL* joined by a NotI site (primers are listed in Table S1). The purpose of including the *amyL* sequence was to achieve homologous recombination of the plasmid into the *amyL* gene of *B. licheniformis*. The complementing sequences were constructed by PCR (ordinary or SLIC) using AccuPrime high-fidelity *Taq* polymerase (Invitrogen) and the primers listed in Table S1. The pMAD vector carrying the complementing sequence was transformed into *B. licheniformis* mutant strains by electroporation (37, 53). The whole plasmid construct was integrated into the chromosome by a single crossover caused by a temperature shift, which influences the temperature-sensitive replicon of pMAD. All complementations were verified by PCR and sequencing.

For *trans* complementation, selected genes were carried by the low-copy-number shuttle vector pHT315 (54). The respective genes and their associated regulatory sequences were amplified by PCR using primers listed in Table S1 and AccuPrime *Taq* DNA polymerase (Thermo Fisher Scientific) according to the manufacturer's instructions. The amplicons were cloned into pHT315, and the resulting constructs were used to transform electrocompetent *B. licheniformis* deletion mutants as described previously (53). The presence of the correct plasmid construct was verified by PCR and sequencing.

Germination assays. Spores were prepared, washed, and stored for at least 7 days prior to use, as described previously (49). All germination assays were performed on pure (>98%), heat-activated (20 min at 65°C) spore suspensions as described previously (49). This purification protocol gives a homogeneous suspension of phase-bright spores without traces of vegetative cells. L-Amino acids (Sigma-Aldrich) were added, and the decrease in the optical density at 600 nm (OD_{600}) of the spore population was monitored. The germination rate was assessed as the rate of phase darkening of the whole spore population, which most likely represents an average rate of individual spore germination. The maximum germination rate (G_{max}) was calculated from the curves obtained from measuring the decrease in the OD_{600} using DMFit (DM is dynamic modeling) (55). Phase-contrast microscopy was also used to monitor the level of germinated spores after 120 min of exposure to germinants. The number of phase-dark (germinated) spores was determined for at least 400 spores in each experiment by counting spores in 10 random fields of view, and the average percentages of germinated spores were calculated from three independent spore batches. Spore suspensions with Milli-Q water were used as negative controls in all germination assays.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00451-19.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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