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Effects of High Pressure on *Bacillus licheniformis* Spore Germination and Inactivation

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ABSTRACT *Bacillus* and *Clostridium* species form spores, which pose a challenge to the food industry due to their ubiquitous nature and extreme resistance. Pressurization at <300 MPa triggers spore germination by activating germination receptors (GRs), while pressurization at >300 MPa likely triggers germination by opening dipicolinic acid (DPA) channels present in the inner membrane of the spores. In this work, we expose spores of *Bacillus licheniformis*, a species associated with food spoilage and occasionally with food poisoning, to high pressure (HP) for holding times of up to 2 h. By using mutant spores lacking one or several GRs, we dissect the roles of the GerA, Ynd, and GerK GRs in moderately HP (mHP; 150 MPa)-induced spore germination. We show that Ynd alone is sufficient for efficient mHP-induced spore germination. GerK also triggers germination with mHP, although at a reduced germination rate compared to that of Ynd. GerA stimulates mHP-induced germination but only in the presence of either the intact GerK or Ynd GR. These results suggests that the effectiveness of the individual GRs in mHP-induced germination differs from their effectiveness in nutrient-induced germination, where GerA plays an essential role. In contrast to *Bacillus subtilis* spores, treatment with very HP (vHP) of 550 MPa at 37°C did not promote effective germination of *B. licheniformis* spores. However, treatment with vHP in combination with elevated temperatures (60°C) gave a synergistic effect on spore germination and inactivation. Together, these results provide novel insights into how HP affects *B. licheniformis* spore germination and inactivation and the role of individual GRs in this process.

IMPORTANCE Bacterial spores are inherently resistant to food-processing regimes, such as high-temperature short-time pasteurization, and may therefore compromise food durability and safety. The induction of spore germination facilitates subsequent inactivation by gentler processing conditions that maintain the sensory and nutritional qualities of the food. High-pressure (HP) processing is a nonthermal food-processing technology used to eliminate microbes from food. The application of this technology for spore eradication in the food industry requires a better understanding of how HP affects the spores of different bacterial species. The present study provides novel insights into how HP affects *Bacillus licheniformis* spores, a species associated with food spoilage and occasionally food poisoning. We describe the roles of different germination receptors in HP-induced germination and the effects of two different pressure levels on the germination and inactivation of spores. This study will potentially contribute to the effort to implement HP technology for spore inactivation in the food industry.

KEYWORDS *Bacillus licheniformis*, spore germination, germination receptor, endospores, high pressure, high-pressure processing, spore inactivation, flow cytometry

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To survive potentially lethal conditions, several members of the orders *Bacillales* and *Clostridiales* form metabolically dormant spores. The spores are much more resistant than vegetative cells to stressors such as UV irradiation, wet and dry heat, freezing, and chemical assaults (1, 3). Many spore-forming bacterial species are associated with foodborne illness and food spoilage, which, combined with their extreme resistance to traditional food-processing strategies, makes them a major concern to the food industry.

Several protective layers surround the dehydrated spore core, including the inner membrane (IM), peptidoglycan cortex, outer membrane, spore coats, crust, and sometimes a sac-like exosporium (2). The spore core contains large amounts of pyridine-2,6-dicarboxylic acid (dipicolinic acid [DPA]) chelated in a 1:1 ratio with Ca^{2+} (CaDPA) and DNA-binding small acid-soluble proteins (SASPs), which also contribute to the extreme resistance of the spores (1, 3). Environmental signals trigger the spores to exit dormancy via the process of germination. These signals include exposure to specific nutrients (germinants) or to nonnutrient factors, such as exogenous CaDPA, cationic surfactants, and high pressures (2, 4–7). Nutrient germinants act on their cognate germination receptors (GRs) present in the spore's IM. The GRs are encoded by *gerA* family genes present in most *Bacillus* and *Clostridium* species (7, 8). The *gerA* genes are often organized in tricistronic operons encoding the A, B, and C subunits, which together compose the functional GR (9, 10). *Bacillus subtilis*, which is a frequently used model organism for studying bacterial spores, contains genes encoding functional GerA, GerK, and GerB GRs and the *ynd* and *yfk gerA* family operons with unknown functions (7). The GerA GR triggers germination when activated by L-valine or L-alanine, whereas the GerB and GerK GRs cooperate to trigger germination in response to a mixture of L-asparagine, glucose, fructose, and K^+ (AGFK) (7, 11). *Bacillus licheniformis*, a close relative of *B. subtilis*, frequently causes spoilage of foods, such as dairy products, bread, and canned meats, and it is also occasionally associated with foodborne disease (12–18). Three functional GRs, GerA, Ynd, and GerK, have been identified in the *B. licheniformis* type strain ATCC 14580/DSM 13 (8, 19–21). We have recently shown that *B. licheniformis* spore germination in response to nutrients depends on cooperation between the GerA GR and the Ynd or GerK GR, and that the most efficient nutrient germinants are L-alanine and L-cysteine (19, 22). Setlow et al. (23) divided the spore germination process into two distinct stages. During the first stage, the GRs are activated by nutrients, followed by a rapid release of the spores' depot of CaDPA, partial rehydration of the core, and partial loss of heat resistance. In the second stage, cortex lytic enzymes (CLEs) degrade the cortex, which leads to increased water influx to the core. As the core rehydrates and expands, metabolism resumes, and the spores enter the outgrowth phase (2, 23). During this process, the spores become fully susceptible to environmental stress (1, 3).

Because of spores' extreme resistance, traditional food-processing methods for spore inactivation often rely on harsh temperature treatments, which have the disadvantage of altering the food's nutritional and sensory qualities. Alternative processing methods, where inactivation can be achieved at lower temperatures, are therefore of interest to food manufacturers. One such technology is high-pressure (HP) processing, which reduces the microbial load without compromising the fresh quality of the food. Conditions of HP in the range of 200 to 600 MPa combined with low to moderate heat are currently used in the food industry to inactivate vegetative microorganisms or to modulate enzymatic reactions in food, but they have not yet been implemented for elimination of spores due to their high-pressure resistance at ambient temperatures (24, 25). To achieve HP inactivation of spores, it is generally accepted that spores must first germinate and that germinated spores are inactivated by subsequent heat or pressure exposure in a two-step process (26, 27). Recently, flow cytometric analysis of HP-treated *B. licheniformis* and *B. subtilis* spores has revealed that HP inactivation of spores is a three-step process, with an "unknown" physiological state between the germinated and the inactivated states (28–30). The mechanism whereby HP initiates germination differs depending on the level of pressure administered. At moderate HP

TABLE 1 Germination and inactivation kinetics for all *B. licheniformis* and *B. subtilis* strains, as determined by plate counts

Strain	Description	Functional GR(s)	HP treatment (MPa)	Temp (°C)	G_{max}[a]	Log germination[b]	Log inactivation[b]
MW3	Wild type	GerA, Ynd, GerK	150	37	0.21 (0.018)	4.9	3.4
			550	37	<0.01	−0.5	−0.6
			550	60	0.82 (0.050)	4.3	4.8
NVH-1032	Food isolate		150	37	0.029 (0.005)	3.3	3.1
			550	37	<0.01	0.01	−0.1
			550	60[c]	0.33 (0.035)	4.8	4.2
PS832	Wild type	GerA, GerB, GerK	150	37	0.26 (0.034)	5.1	4.3
			550	37	0.054 (0.007)	2.5	0.3
			550	60[c]	0.98 (0.007)	5.3	5.2
NVH-1307	Δ*gerAA* mutant	Ynd, GerK	150	37	0.17 (0.040)	3.6	1.0
NVH-1335	Δ*yndD* mutant	GerA, GerK	150	37	0.071 (0.028)	3.4	2.3
NVH-1324	Δ*gerKA-C* mutant	GerA, Ynd	150	37	0.20 (0.021)	4.9	2.9
NVH-1323	Δ*gerAA* Δ*gerKA-C* mutant	Ynd	150	37	0.16 (0.040)	2.7	1.2
NVH-1368	Δ*gerAA* Δ*yndD* mutant	GerK	150	37	<0.01	0.7	−1.8
NVH-1376	Δ*yndD* Δ*gerKA-C* mutant	GerA	150	37	<0.01	0.05	−0.3
NVH-1370	Δ*gerAA* Δ*yndD* Δ*gerKA-C* mutant	None	150	37	<0.01	−0.4	−0.6

[a]G_{max}, maximum germination rate (log heat-sensitive spores per minute). Standard error of the estimate (SE_{est}) is presented in parentheses.
[b]Log germinated or log inactivated spores after HP treatment determined by plate counts. Data are presented as arithmetic means.
[c]Only one spore batch.

(mHP) of 80 to 300 MPa, germination is triggered via the GRs, while very HP (vHP) of 300 to 600 MPa triggers GR-independent germination by directly opening the DPA channels causing CaDPA release (27, 31, 32). During vHP (>300 MPa) treatment, *B. subtilis* spores become heat sensitive but are not inactivated by the pressure treatment (33). High-pressure thermal sterilization (HPTS) is a strategy that exploits the synergetic effect of vHP (>500 MPa) and elevated temperatures (>60°C) on spore inactivation (24, 34). However, due to technical limitations, temperature inhomogeneity in industrial-scale vessels, and difficulties predicting spore inactivation kinetics, this method is not yet applied commercially (24, 25).

Currently, studies on the effect of HP on GR-induced spore germination have been limited to *B. subtilis* and *Bacillus cereus* spores (31, 36, 37). This study examines the effect of mHP on *B. licheniformis* mutant spores deficient in one or several of the GerA, Ynd, or GerK GRs. The effect of vHP at moderate and elevated temperature on *B. licheniformis* spores was also assessed.

RESULTS

Effect of mHP on spore germination. To explore the effect of mHP on *B. licheniformis* spores, we exposed 10^8 spores/ml of strain MW3 and the slow-germinating food isolate NVH-1032 to 150 MPa at 37°C and determined the level of germination and inactivation by plate counts. For comparison, spores of the *B. subtilis* strain PS832 were subjected to the same treatment. The results are presented as log germinated or inactivated spores (arithmetic mean of two or more spore batches). The exposure of MW3 and PS832 spores to mHP induced spore germination at a maximum rate (G_{max}) of 0.21 and 0.26 log spores/min. After a holding time of 2 h, 4.9 and 5.1 log germinated (heat-sensitive) spores were detected for strains MW3 and PS832, respectively (Table 1 and Fig. 1A and C). The MW3 and PS832 inactivation levels after 2 h of mHP treatment were 3.4 and 4.3 log, respectively (Table 1 and Fig. 1A and C). Spores of strain NVH-1032 displayed a germination rate of 0.029 log spores/min, which was lower than the G_{max} of MW3 and PS832 spores (Table 1 and Fig. 1B). However, NVH-1032 spores treated with mHP for 60 min and 2 h demonstrated a large (up to 3-log) difference between spore batches in the levels of germinated and inactivated spores (Fig. 1B, and Fig. S1 in the supplemental material). Notably, the germination rates were based on an average of the data collected over the first 15 min of mHP exposure, where there was little variation between spore batches.

Flow cytometry analysis of mHP-treated *B. licheniformis* spores. Flow cytometry (FCM) analysis provides information about the physiological state of the HP-treated

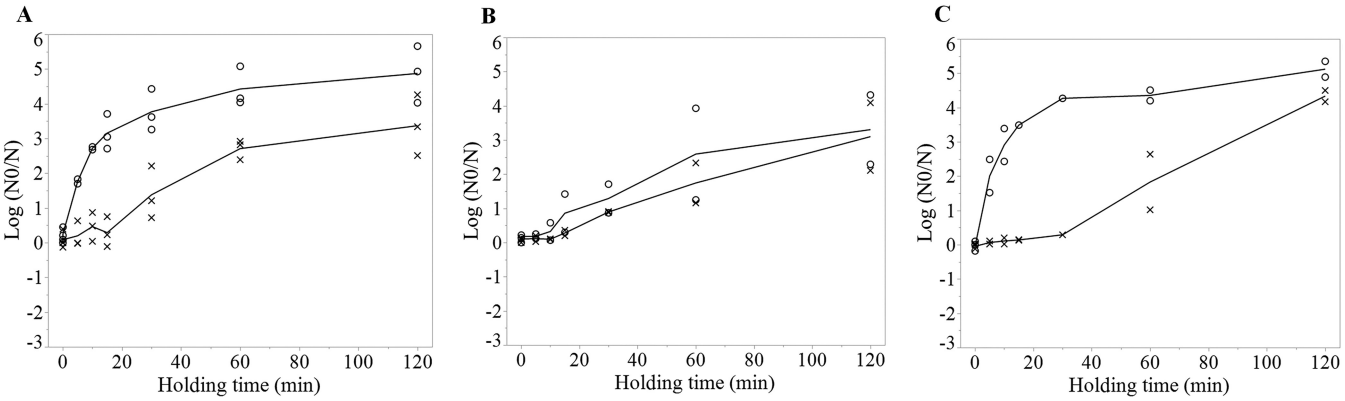

FIG 1 Plate count data showing the effect of mHP treatment (150 MPa at 37°C) on *Bacillus* spores. ○, germinated spores; ×, inactivated spores. Each symbol represents an independent spore batch. (A) *B. licheniformis* strain MW3. (B) *B. licheniformis* strain NVH-1032. (C) *B. subtilis* strain PS832.

spores. By studying staining characteristics of MW3 spore samples containing known physiological states, five subpopulations representing dormant (R1), germinated and viable (R2), unknown (R3 and R4), and inactivated (R5) states were identified (Fig. 2). The percentages of spores detected in each physiological state are shown in Table S1. The dormant spore population displayed low fluorescence intensities for both propidium iodide (PI) and Syto16 (Fig. 2, R1). As the spores germinated, more Syto16 entered the core of the spores, which increased the Syto16 fluorescence intensity, while the PI fluorescence intensity stayed low (Fig. 2, R2). At mHP holding times ≥5 min, two other subpopulations, both displaying staining characteristics different from those of dormant and germinated spores, started to emerge between R1 and R2. The subpopulation in the first "unknown" state (R3) displayed a lower Syto16 fluorescence intensity than the germinated spores, but the PI fluorescence intensity did not change. The second subpopulation (R4), which increased in size with longer holding times, displayed even lower Syto16 fluorescence intensity than the subpopulation in R3 (Fig. 2, R4). Heat treatment after HP exposure resulted in a 5th subpopulation that exhibited a strong PI fluorescence intensity indicative of damage to the IM of the spores (Fig. 2, R5). The great majority of the spore populations were present in the R5 region after an additional heat treatment following HP exposure, which suggests that the spores present in R2, R3, and R4 were heat sensitive (had germinated). When we compare FCM data and plate count data below, we have interpreted the spores in regions R2 to R4, as well as R5, as spores that have undergone germination and now possess different degrees of injuries (sublethal to lethal).

Sensitivity of individual germination receptors to mHP. To assess the role of individual GRs in mHP-induced germination, we exposed spores of MW3 *ger* double-null mutants to mHP. Spores of strain NVH-1323 (Δ*gerAA* and Δ*gerKA* Δ*gerKC* [Δ*gerKA*-

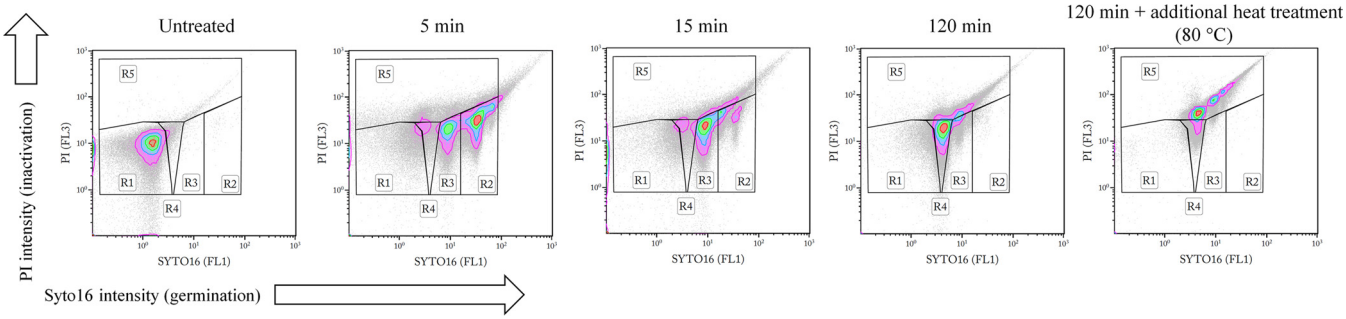

FIG 2 Contour-density plots of FCM data showing the effect of mHP treatment (150 MPa at 37°C) on the physiological state of *B. licheniformis* strain MW3 spores. Gating: R1, dormant spores; R2, germinated spores; R3, unknown state 1; R4, unknown state 2; and R5, inactivated spores. Contour-density plots depict the results from analyses performed on the CyFlow ML (~200,000 events). Instrument-specific gates were constructed as described in "Flow cytometry analysis of mHP-treated *B. licheniformis* spores," above.

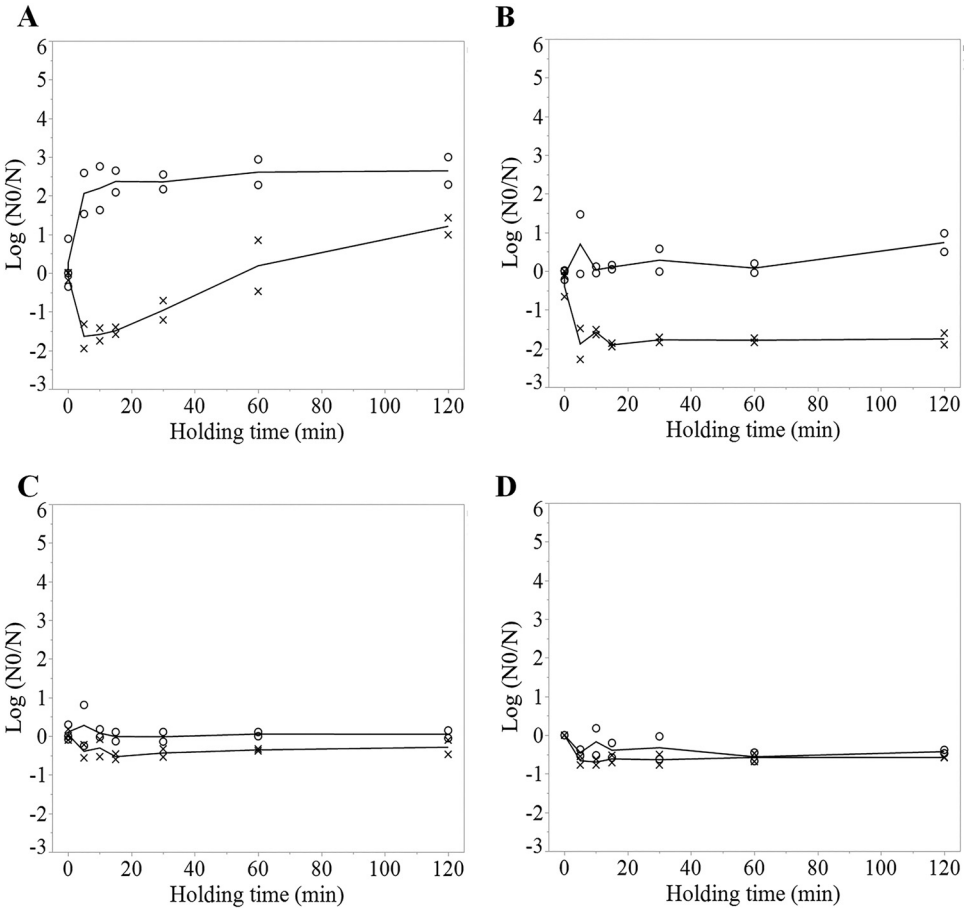

FIG 3 Plate count data showing the effect of mHP treatment (150 MPa at 37°C) on spores of *B. licheniformis ger* double- and triple-null mutants. ○, germinated spores; ×, inactivated spores. Each symbol represents an individual spore batch. (A) NVH-1323 (Δ*gerAA* Δ*gerKA-C*). (B) NVH-1368 (Δ*gerAA* Δ*yndD*). (C) NVH-1376 (Δ*gerKA-C* Δ*yndD*). (D) NVH-1370 (Δ*gerAA* Δ*gerKA-C* Δ*yndD*).

C]), in which the only intact GR expressed is Ynd (Ynd$^+$), demonstrated a G_{max} comparable to that of wild-type (MW3) spores (0.16 log spores/min compared to 0.21 log spores/min, respectively). Two hours of mHP treatment of NVH-1323 (Ynd$^+$) spores resulted in 2.7 log germination and 1.2 log inactivation (Table 1 and Fig. 3A), which was 2.2 log lower, with respect to both germination and inactivation, than observed for wild-type spores (Table 1 and Fig. 1A). This was also confirmed by FCM analysis (Fig. 4). NVH-1368 spores (Δ*gerAA* Δ*yndD*), in which the only intact GR expressed is GerK (GerK$^+$), also demonstrated a reduced level of germination (0.7 log) compared to the wild-type and NVH-1323 (Ynd$^+$) spores after 2 h of treatment (Table 1 and Fig. 3B). Inactivation of NVH-1368 (GerK$^+$) spores was −1.8 log after 2 h of treatment. The negative value for log inactivated spores is due to an increase in CFU per milliliter after mHP treatment compared to the untreated sample (N_0). This was also observed for other strains and resulted in a "dip" in the curves in Fig. 3A and B and 5A. FCM analyses of NVH-1368 (GerK$^+$) spores showed that a subpopulation germinated in response to mHP (Fig. 4). After 15 min of treatment, approximately 57% of the total population had germinated (entered R2, R3, R4, and R5 states), compared to approximately 94% and 89% of the wild-type and NVH-1323 (Ynd$^+$) spore populations, respectively (Fig. 4). After 2 h of mHP exposure, the majority of the NVH-1368 spore population had germinated (Fig. 4). For spores of strain NVH-1376 (Δ*gerKA-C* Δ*yndD*), in which the only intact GR expressed is GerA (GerA$^+$), and spores of strain NVH-1370 (Δ*gerAA* Δ*gerKA-C* Δ*yndD*), which lack all functional GRs (Ger$^-$), no mHP-induced germination was detected, as determined by the plate counts (Table 1 and Fig. 3C and D). However, it

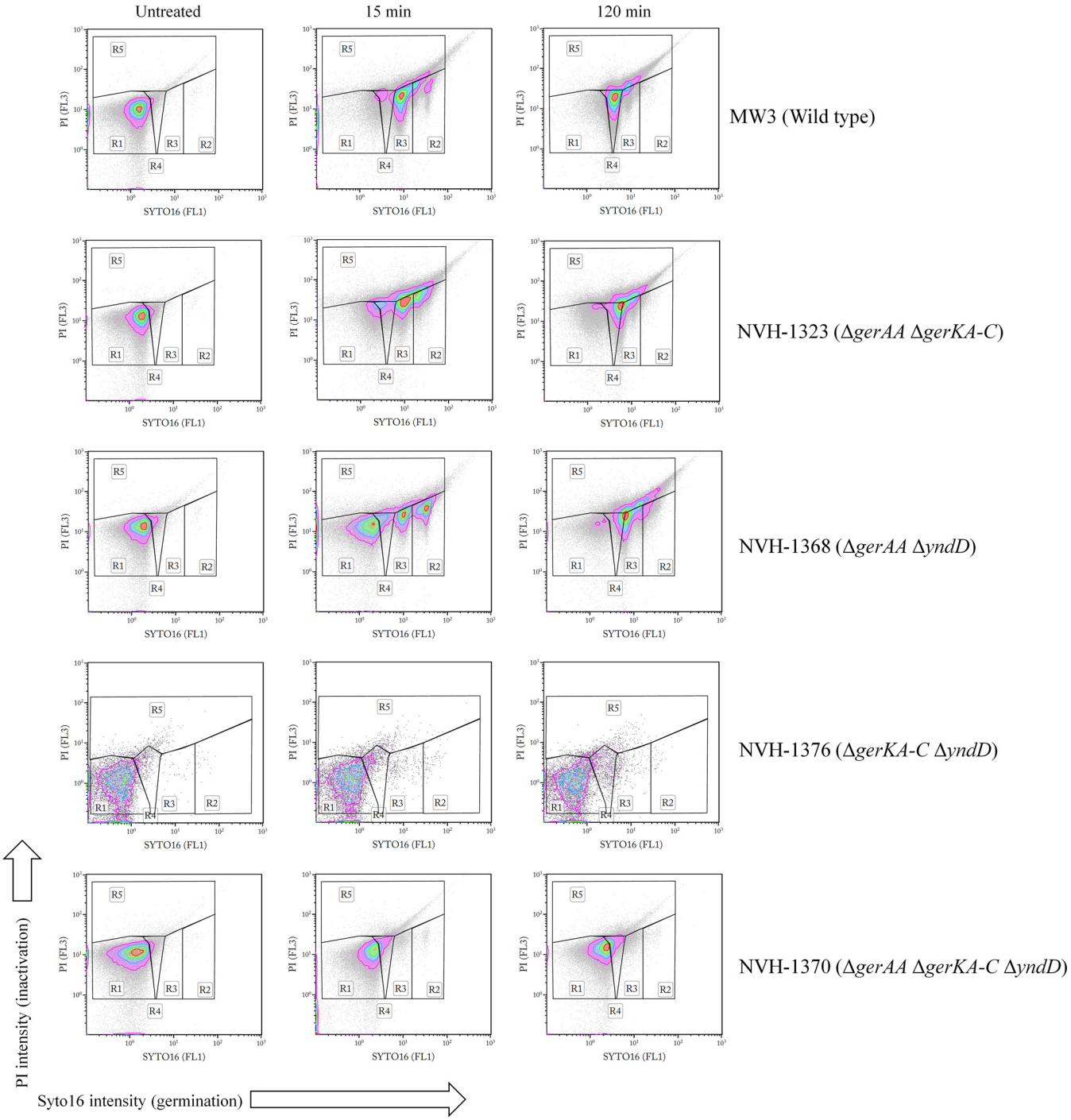

FIG 4 Contour-density plots of FCM data showing the effect of mHP treatment (150 MPa at 37°C) on the physiological state of wild-type and *ger* double- and triple-null mutant spores of the *B. licheniformis* strain MW3. Gating: R1, dormant spores; R2, germinated spores; R3, unknown state 1; R4, unknown state 2; R5, inactivated spores. Contour-density plots depict the results from analyses of MW3 (wild type), NVH-1323 (Δ*gerAA* Δ*gerKA-C*), NVH-1368 (Δ*gerAA* Δ*yndD*), and NVH-1370 (Δ*gerAA* Δ*gerKA-C* Δ*yndD*) spores performed on the CyFlow ML (~200,000 events). NVH-1376 (Δ*gerKA-C* Δ*yndD*) analyses were performed on the FACSCalibur (~10,000 events). Instrument-specific gates constructed as described in "Flow cytometry analysis of mHP-treated *B. licheniformis* spores," above.

should be noted that the NVH-1370 (Ger$^-$), NVH-1376 (GerA$^+$), and NVH-1368 (GerK$^+$) spores were still able to germinate and form colonies on nutrient agar, as approximately 0.1 to 1% of the total spore population germinated (data not shown). FCM analyses confirmed that NVH-1376 (GerA$^+$) and NVH-1370 (Ger$^-$) spores did not germinate in response to mHP treatment (Fig. 4).

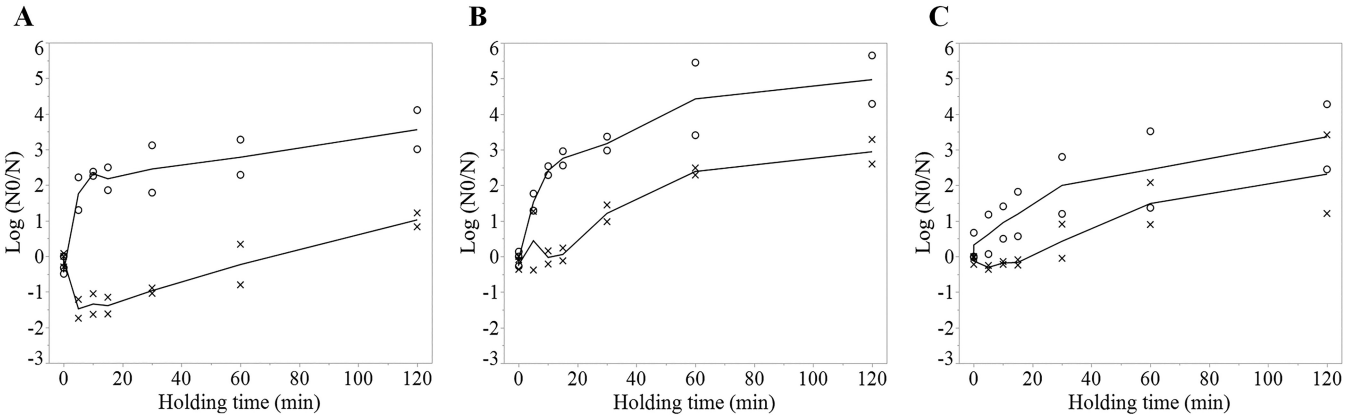

FIG 5 Plate count data showing the effect of mHP treatment (150 MPa at 37°C) on *B. licheniformis ger* single-null mutant spores. ○, germinated spores; ×, inactivated spores. Each symbol represents an individual spore batch. (A) NVH-1307 (Δ*gerAA*), (B) NVH-1324 (Δ*gerKA-C*), and (C) NVH-1335 (Δ*yndD*).

To address the possibility of functional cooperation between GRs in mHP-induced germination, we exposed spores of *ger* single-null mutant strains to mHP. Spores of strain NVH-1307 (Δ*gerAA* mutant), expressing intact GerK and Ynd GRs (GerK$^+$ Ynd$^+$), and spores of strain NVH-1324 (Δ*gerKA-C* mutant) expressing intact GerA and Ynd GRs (GerA$^+$ Ynd$^+$), displayed G_{max} values in response to mHP similar to spores of the wild-type strain, i.e., 0.17 log spores/min and 0.20 log spores/min compared to 0.21 log spores/min, respectively (Table 1 and Fig. 5A and B, compared to Fig. 1A). In contrast, spores of strain NVH-1335 (Δ*yndD* mutant), which expresses intact GerA and GerK GRs (GerA$^+$ GerK$^+$), demonstrated a reduced G_{max} (0.071 log spores/min) compared to the wild type, NVH-1307 (GerK$^+$ Ynd$^+$), and NVH-1324 (GerA$^+$ Ynd$^+$) spores (Table 1 and Fig. 5C). FCM results confirmed the plate counts, as a larger fraction of the NVH-1335 (GerA$^+$ GerK$^+$) spore population remained dormant (R1) after mHP exposure while large germinated subpopulations (R2, R3, and R4) were seen in the wild-type, NVH-1324 (GerA$^+$ Ynd$^+$), and NVH-1307 (GerK$^+$ Ynd$^+$) spore populations (Fig. S3).

Germination and inactivation of spores by vHP. In order to obtain information about the effect of vHP on *B. licheniformis* spores, spores of strains MW3 and NVH-1032 were exposed to 550 MPa at 37°C for holding times of up to 2 h. *B. subtilis* PS832 was included for comparison. Exposure to vHP did not trigger germination or inactivation of MW3 and NVH-1032 spores, even after a holding time of 2 h, as determined by plate counting (Table 1 and Fig. 6A and B). FCM analyses of NVH-1032 spores treated with vHP confirmed the plate count results (Fig. S4). However, FCM analyses of spores of strain MW3 revealed a subpopulation of germinated spores following this treatment

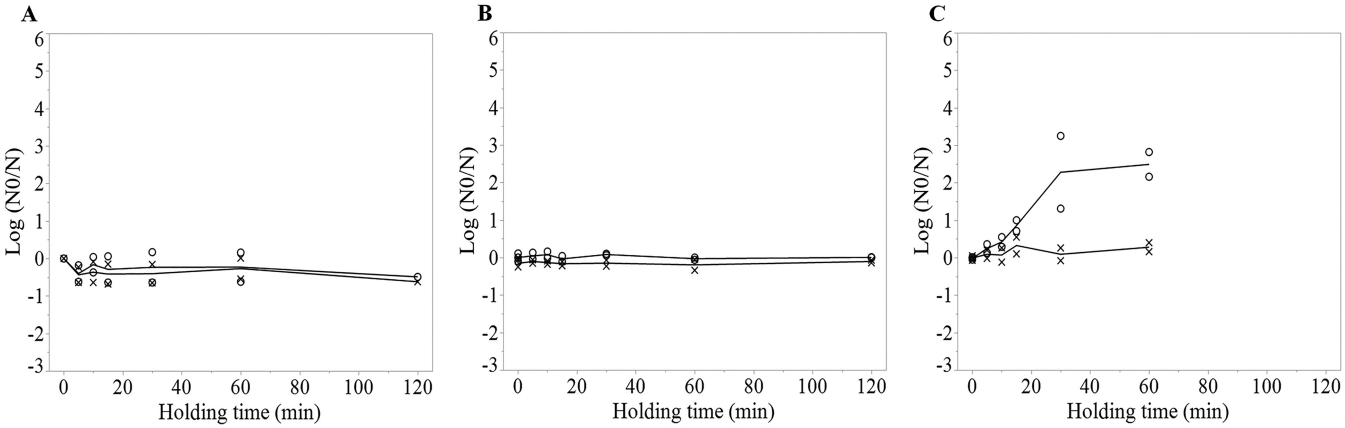

FIG 6 Plate count data showing the effect of vHP treatment (550 MPa at 37°C) on *Bacillus* spores. ○, germinated spores; ×, inactivated spores. Each symbol represents an individual spore batch. (A) *B. licheniformis* strain MW3. (B) *B. licheniformis* strain NVH-1032. (C) *B. subtilis* strain PS832.

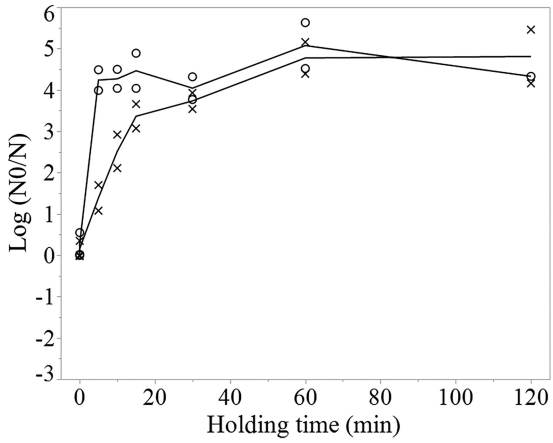

FIG 7 Plate count data showing the effect of vHP treatment (550 MPa at 60°C) on *B. licheniformis* strain MW3 spores. Each symbol represents an individual spore batch. ○, germinated spores; ×, inactivated spores.

(Fig. S4), which corresponded to approximately 58% of the total spore population after 2 h of vHP treatment (Fig. S4). Germination at this level can be difficult to detect accurately by plate counts, as it corresponds to ~0.4 log germinated spores. *B. subtilis* PS832 spores demonstrated 2.5 log germination after 60 min of vHP exposure, while 0.3 log spore inactivation was detected (Table 1 and Fig. 6C). This was also supported by FCM analyses. However, the formation of subpopulations in R3 and R4 following germination (R2) was slower than when spores were exposed to mHP (Fig. S4).

To study the effect of vHP combined with high temperature on spore germination and inactivation, spores of strain MW3 were exposed to 550 MPa at 60°C for holding times of up to 2 h. This treatment resulted in 4.3 log germination and 4.8 log inactivation after 2 h of vHP exposure (Table 1 and Fig. 7). In accordance with these results, FCM analyses showed that a large proportion of the spore population was inactivated after 2 h of exposure, i.e., displayed strong PI fluorescence intensities (Fig. S6). However, it should be noted that the DNA-binding abilities of Syto16 and PI are reduced in spores treated under these conditions compared to the HP treatments at 37°C (29, 30, 35). Similar results were obtained for spores of the *B. subtilis* strain PS832, as determined by plate counting (Table 1 and Fig. S5B). Spores of the *B. licheniformis* strain NVH-1032 exhibited lower G_{max} (0.33 log spores/min) than the spores of strains MW3 and PS832 (0.82 and 0.98 log spores/min, respectively) in response to this treatment (Table 1). However, after 2 h of exposure, the total levels of germinated and inactivated NVH-1032 spores were 4.8 log and 4.2 log, respectively, which is similar to the levels observed for strains MW3 and PS832 (Table 1 and Fig. S5A).

DISCUSSION

In the present study, we have investigated the effects of mHP and vHP on *B. licheniformis* spores and assessed the contribution of individual GRs to mHP-induced germination. We found that mHP treatment at 37°C induced *B. licheniformis* spore germination via the GerA, GerK, and Ynd GRs. The Ynd GR appeared to play the central role in mHP-induced germination, as spores of strain NVH-1323 (Ynd^+) germinated with an efficiency similar to that of the wild-type spores. In addition, NVH-1335 ($GerA^+$ $GerK^+$) spores, which carry a *yndD* deletion, demonstrated reduced germination efficiency. Spores of NVH-1368, expressing only GerK, also germinated in response to mHP, although not as efficiently as NVH-1323 (Ynd^+) spores or wild-type spores. The importance of the Ynd and GerK GRs in mHP-induced spore germination was supported by the inability of strain NVH-1376 ($GerA^+$) spores to germinate in response to mHP exposure. Spores of strain NVH-1335 ($GerA^+$ $GerK^+$) demonstrated a more efficient germination response to mHP than NVH-1368 ($GerK^+$) spores, and similarly, NVH-1324

(GerA$^+$ Ynd$^+$) spores demonstrated a more efficient germination response than NVH-1323 (Ynd$^+$) spores. This suggests that GerA facilitates mHP-induced germination in the presence of the GerK or the Ynd GR. This is similar to the cooperation between GerA, Ynd, and GerK GRs during nutrient-induced germination of *B. licheniformis* spores (19).

We have recently shown that the GerA GR appears to play the most prominent role in nutrient-induced germination (19). Gene depletions in the *gerA* operon were more detrimental to nutrient-induced germination than gene depletions in the *ynd* and *gerK* operons (19, 22). This differs from the role of the individual GRs in mHP-induced germination, as revealed by the results from the present study, where Ynd appeared to play a more prominent role than the other GRs. In *B. licheniformis*, the individual GRs' contribution to nutrient- and mHP-induced germination observed for *B. licheniformis* spores differs from that observed for *B. subtilis* spores, where the individual GRs' mHP responsiveness corresponds to their relative contribution to nutrient-induced germination (7, 37). However, a study on how mHP affects *B. cereus* spores lacking one of the seven GRs showed that loss of individual GRs did not affect mHP-induced germination (36). The reason for the differences in mHP sensitivity between GRs is not fully understood. It has previously been shown that the rate of mHP-induced germination can be increased by overexpressing individual *ger* operons (37, 38). We have shown that *yndD* and *gerKA* are expressed at ~10 times higher levels than *gerAA* during sporulation (19). If the expression levels reflect the levels of GRs in the IM, this could at least partly explain the importance of Ynd and GerK in mHP-induced germination. In addition to GR levels in the spores' IM, structural properties of the GR might play a role in its mHP responsiveness. In *B. subtilis*, variants of the GerB GR with single amino acid substitutions in the A or B subunits were more responsive to mHP than the native GerB GR (37). The gene organization of the *ynd* operon in *B. licheniformis* differs from that typically found in *Bacillus* species, as it contains three B subunit genes, in contrast to the more commonly occurring single B subunit gene (8, 20, 21). The potential significance of the multiple B subunits for the sensitivity to mHP and nutrient-induced germination is currently unknown but will be subjected to further studies.

Exposure to 550 MPa (vHP) at 37°C induced only low levels of germination of *B. licheniformis* spores, while *B. subtilis* spores germinated more efficiently under these conditions. The absence of substantial spore inactivation under these conditions has previously been described for *B. subtilis* spores (29), and this was confirmed in our study. The difference in vHP responsiveness between *B. licheniformis* and *B. subtilis* spores could be due to structural differences in the SpoVA proteins that form the mechanosensitive DPA channels (39). Alternatively, vHP could inactivate other proteins or pathways responsible for CaDPA release and subsequent germination. Differences in the IM, for instance, in the fatty acid composition, may also influence the sensitivity of the spores to vHP. However, in previous studies, spores of *B. subtilis* strains with altered levels of unsaturated fatty acids in the IM demonstrated no difference in vHP responsiveness compared to wild-type spores (32). Sporulation temperature has been shown to affect spore germination in response to HP, likely by affecting the fluidity of the IM. *B. subtilis* spores produced at higher temperatures displayed reduced vHP-induced germination rates, whereas mHP-induced germination rates increased with increased sporulation temperatures (32, 37). *B. licheniformis* spores have previously been reported to germinate in response to 600 MPa at 77°C (30), as high temperature and HP are known to act synergistically on germination and inactivation (24). In accordance with that study (30), we observed efficient germination and inactivation of MW3 and NVH-1032 spores after they were exposed to vHP at 60°C.

In this study, some of the *B. licheniformis* strains showed an approximately 100-fold increase in CFU per milliliter after mHP treatment, as detected by the plate counts. A similar effect has been described before, as a "shoulder formation" in the inactivation kinetics of HP-treated *B. subtilis* spores, a phenomenon that was assigned to spore agglomerates that disassociated during HP treatment (40, 41). In the present study, we observed an increase in CFU per milliliter for three strains (Fig. 3A and B and 5A). However, the FCM analyses supported the plate count data on germinated (heat-

sensitive) spores for these strains, and the shoulder formation observed in the inactivation kinetic curves was not investigated further. In our experiments, some variation in mHP responsiveness between spore batches was observed; particularly, the food isolate NVH-1032 demonstrated a large variation between the two spore batches used. Both sporulation conditions and stochastic events during sporulation have been shown to affect different spore properties, including germination behavior (42). In addition, heterogeneity in germination and inactivation behavior between spores of different species, strains, spore batches, and even within the same population has been described before (26, 42–44).

Approximately 0.1 to 1% of *B. licheniformis* spores lacking all functional GRs were still capable of forming colonies on nutrient agar. Similar observations have been made in *B. subtilis* and were attributed to spontaneous germination (7). Spontaneous germination may also explain our observations. There is an orphan *gerA* homolog (*yndF2*) encoding an N-terminally truncated C subunit in the *B. licheniformis* type strain ATCC 14580/DSM 13 genome (20, 21). However, deletion of this gene did not significantly affect L-alanine or L-cysteine-induced germination (our unpublished data) and is therefore not likely to explain the low level of germination observed in spores lacking all functional GRs.

The results from the FCM analyses largely supported the observations from the plate count data, and they also provided information about the physiological state of the spores. The FCM analyses identified two distinct subpopulations representing unknown states, which increased with increasing HP holding times. Previous FCM analyses of HP-treated *B. subtilis* and *B. licheniformis* spores have detected one such unknown state (28, 30, 40). The two unknown states seen in the present study displayed lower Syto16 fluorescence intensities than the germinated subpopulation. Mathys et al. (30) hypothesized that the lower Syto16 fluorescence in the unknown state could be due to the entry of some PI into the core, which partly displaces Syto16 or leads to quenching of Syto16 by fluorescence resonance energy transfer to PI. Syto16 is commonly used in eukaryotic cells as an indicator for apoptosis, as Syto16 fluorescence intensity is lost during cell death (45, 46). A loss of Syto16 fluorescence upon programmed cell death has also been reported for *B. subtilis* mother cells during sporulation (47). We compared data from the FCM analyses and plate counts to assess the viability of the spores in the two unknown states. After 15 min of mHP exposure of *B. licheniformis* wild-type spores, the FCM analyses showed a large subpopulation in R3, and the plate counts showed a high level of germinated (heat-sensitive) spores and low levels of inactivation. After 2 h of mHP exposure, FCM analyses showed a large subpopulation in R4, and the plate counts showed a high level of inactivation. This suggests that the subpopulation present in region R3 (unknown state 1) corresponds to viable spores capable of outgrowth on nutrient agar, whereas the subpopulation present in region R4 (unknown state 2) likely represents uncultivable spores. Syto16 does not bind exclusively to DNA, at least in eukaryotic cells, but also binds to cytoplasmic contents (46). The loss of Syto16 fluorescence could be due to DNA fragmentation, breakdown of cytoplasmic contents, or membrane leakage of cytoplasmic contents (45). As PI fluorescence intensities are low, even after prolonged HP treatment at both mHP and vHP (37°C), extensive membrane disruptions are unlikely. Therefore, the unknown regions likely correspond to spores with different degrees of injury. However, further studies using cell sorting are needed to better understand the physiological characteristics of HP-exposed spores.

MATERIALS AND METHODS

Strains and spore preparation. The strains included in this study are listed in Table 2. The *B. licheniformis* strain NVH-1032 was isolated from a batch of spoiled canned meat, and its spores have been shown to germinate significantly more slowly than spores of the type strain *B. licheniformis* DSM 13 (50). *B. licheniformis* strain MW3 is a more readily transformable derivative of the type strain (51). The *B. licheniformis ger*-null mutants are isogenic to MW3 and carry different in-frame deletions in the *gerA* family operons (19, 22). The *B. subtilis* strain PS832 is a *trp*$^+$ derivative of *B. subtilis* 168 (7). Spores of the *B. licheniformis* strains and *B. subtilis* PS328 were prepared as described previously (7, 50, 52). Sporulation of all strains was done at 37°C, and spores were stored in sterile water.

TABLE 2 Strains used in this work

Strain or isolate	Description	Reference
PS832	*B. subtilis* subsp. *subtilis* 168 *trp*$^+$	7
NVH-1032	*B. licheniformis* food isolate	55
MW3	*B. licheniformis* DSM 13 Δ*hsdR1* Δ*hsdR2*	51
NVH-1307	*B. licheniformis* MW3 Δ*gerAA*::*spc*	22
NVH-1323	*B. licheniformis* MW3 Δ*gerAA*::*spc* Δ*gerKA-C*	19
NVH-1324	*B. licheniformis* MW3 Δ*gerKA-C*	19
NVH-1335	*B. licheniformis* MW3 Δ*yndD*	19
NVH-1368	*B. licheniformis* MW3 Δ*gerAA*::*spc* Δ*yndD*	19
NVH-1370	*B. licheniformis* MW3 Δ*gerAA*::*spc* Δ*gerKA-C* Δ*yndD*	19
NVH-1376	*B. licheniformis* MW3 Δ*gerKA-C* Δ*yndD*	19

High-pressure treatment. For HP treatment of spores, a monovessel and a multivessel U111 unit (Unipress, Warsaw, Poland) were used. The multivessel unit had a compression rate of 12 MPa/s, whereas the hand-pump-driven monovessel had a compression rate of 20 to 25 MPa/s. The monovessel unit had a vessel volume of 3.7 ml, the pressure-transmitting medium used was di-2-ethyl-hexyl-sebacate, and 4 300-μl spore solutions were treated at the same time. The multivessel unit had five chambers with vessel volumes of 4 ml each, and these were used for the treatment of four spore samples at a time. The pressure-transmitting medium used in this system was silicon oil. Exposure of spores to sublethal temperatures before induction of germination with nutrients or high pressure is known to synchronize the germination response (53, 54). Therefore, *B. licheniformis* and *B. subtilis* spores were heat activated at 65°C for 20 min prior to HP treatment. Subsequently, the spores were resuspended in 50 mM *N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES) buffer (pH 7.0), and the optical density at 600 nm (OD_{600}) was adjusted to correspond to a spore titer of 10^8 spores/ml. For the experiments using the monovessel unit, a volume of 300 μl of the spore suspension was transferred to a shrinking tube with an inner diameter of 3 mm and outer diameter of 3.6 mm (Schrumpfschlauch 3/1; DSG-Canusa, Meckenheim, Germany), and the tube was sealed with a soldering iron. Four shrinking tubes were packed into a 1.8-ml Nunc cryotube with a screw top (Nunc A/S, Roskilde, Denmark), and the tube was filled with water. The temperature was measured in the tube's geometrical center. For the multivessel unit experiments, spore suspensions were added to 1.6-ml Nunc cryotubes, ensuring that no air bubbles were present, and treated directly with HP. Temperature measurement was done in a dummy sample containing only water, which was HP treated together with the spore samples. A thermocouple (Unipress, Warsaw, Poland) was used to monitor temperatures in both units.

HP experiments were performed at 37°C and 60°C, and the pressure chambers were submerged in a thermostatic bath (cc2; Huber GmbH, Germany) with silicon oil. The HP treatments were performed at 150 MPa and at 550 MPa, with holding times ranging from 1 s to 2 h under isothermal conditions. The HP experiments were performed in technical duplicates using at least two independent spore batches.

Enumeration of inactivated and germinated spores. Following HP treatment, an aliquot of the spore suspension was diluted in Ringer's solution (Merck, Germany), and volumes of 50 μl or 100 μl were plated on Nutrient agar (Oxoid, England) and incubated overnight at 37°C before colony enumeration. The level of spore inactivation was calculated as the log N_0/N ratio of CFU per milliliter of untreated spores (N_0) and CFU per milliliter of the HP-treated spores (N). In each experiment, an aliquot of the HP-treated spore suspension was heat treated at 80°C for 20 min before plating to determine the level of heat-sensitive (germinated) spores. The level of spore germination was calculated using $\log(N_0/N)$, where N_0 is the CFU per milliliter of untreated spores and N is the CFU per milliliter of heat-treated spores. To show the batch variation, the results for each individual spore batch were plotted, with a line representing the mean of the spore batches. The maximum rate of germination (G_{max}) was determined by linear regression of the steepest segment (between holding times of 0 to 15 min) of the germination plots. The lower limit of detection of G_{max} was set to 0.01 log spores/min. The data were analyzed using JMP Pro 12 (SAS Institute Inc., NC, USA). Plate count results from experiments using the two different HP units (multivessel and monovessel) are presented in the same plots, as the differences in HP effects on spore germination and inactivation between the two units were less than the differences observed between spore batches (results not shown).

Flow cytometry analyses. Flow cytometry (FCM) analysis provides information about the physiological state of the spores and was performed as described by Mathys et al. (30). Spore suspensions were double stained with the fluorescent DNA stains Syto16 (Invitrogen, CA, USA) and propidium iodide (PI) (Invitrogen, CA, USA) to distinguish between spore germination and inactivation. Syto16 is membrane permeant but cannot penetrate the spore core, and it is used to indicate breakdown of the cortex during spore germination (32, 37). PI is not membrane permeant and indicates IM rupture and spore inactivation (30). Syto16 and PI were added to the spore suspension at concentrations of 0.5 μM and 15 μM, respectively, and the spores were stored in the dark for 15 min at room temperature prior to FCM analysis. The analyses were carried out with either a CyFlow ML (Partec GmbH, Münster, Germany) or a FACSCalibur flow cytometry instrument (BD Biosciences, CA, USA). HP-treated and untreated spores were diluted in ACES buffer (0.05 M [pH 7.0]) to allow a flow rate of 2,000 events/s, and a total of ~200,000 events were measured for the CyFlow ML, or 1,000 events/s and a total of 10,000 events were measured for the FACSCalibur. The data were analyzed with the Kaluza 3.1 software (Beckman Coulter, USA), using gating strategies based on biological controls confirmed by plate counts and phase-contrast microscopy. The flow cytometry results for a single spore batch are presented in the Results.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00503-17.

SUPPLEMENTAL FILE 1, PDF file, 2.0 MB.

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Correction for Borch-Pedersen et al., "Effects of High Pressure on *Bacillus licheniformis* Spore Germination and Inactivation"

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Volume 83, no. 14, e00503-17, https://doi.org/10.1128/AEM.00503-17. The strain NVH-1376, which was presented as a Δ*yndD* Δ*gerKA-C* double mutant, was found to contain a spectinomycin resistance cassette inserted into the *gerAA* gene, making it a *ger*-null mutant.

Hence, there is no evidence indicating that the GerA germinant receptor cannot function alone in inducing germination in response to moderately high pressures, as was originally argued in our published paper. All statements in the Abstract, Results, and Discussion that GerA cannot induce germination in response to moderately high pressure when present as the single, intact germination receptor should be disregarded.

Page 1, Abstract, line 11: "GerK also triggers" should read "GerK and GerA also trigger."

Page 1, Abstract, lines 12 and 13: The sentence beginning with "GerA stimulates. . ." should be deleted.

Page 2, line 30: "*B. licheniformis* spore germination" should read "efficient *B. licheniformis* germination."

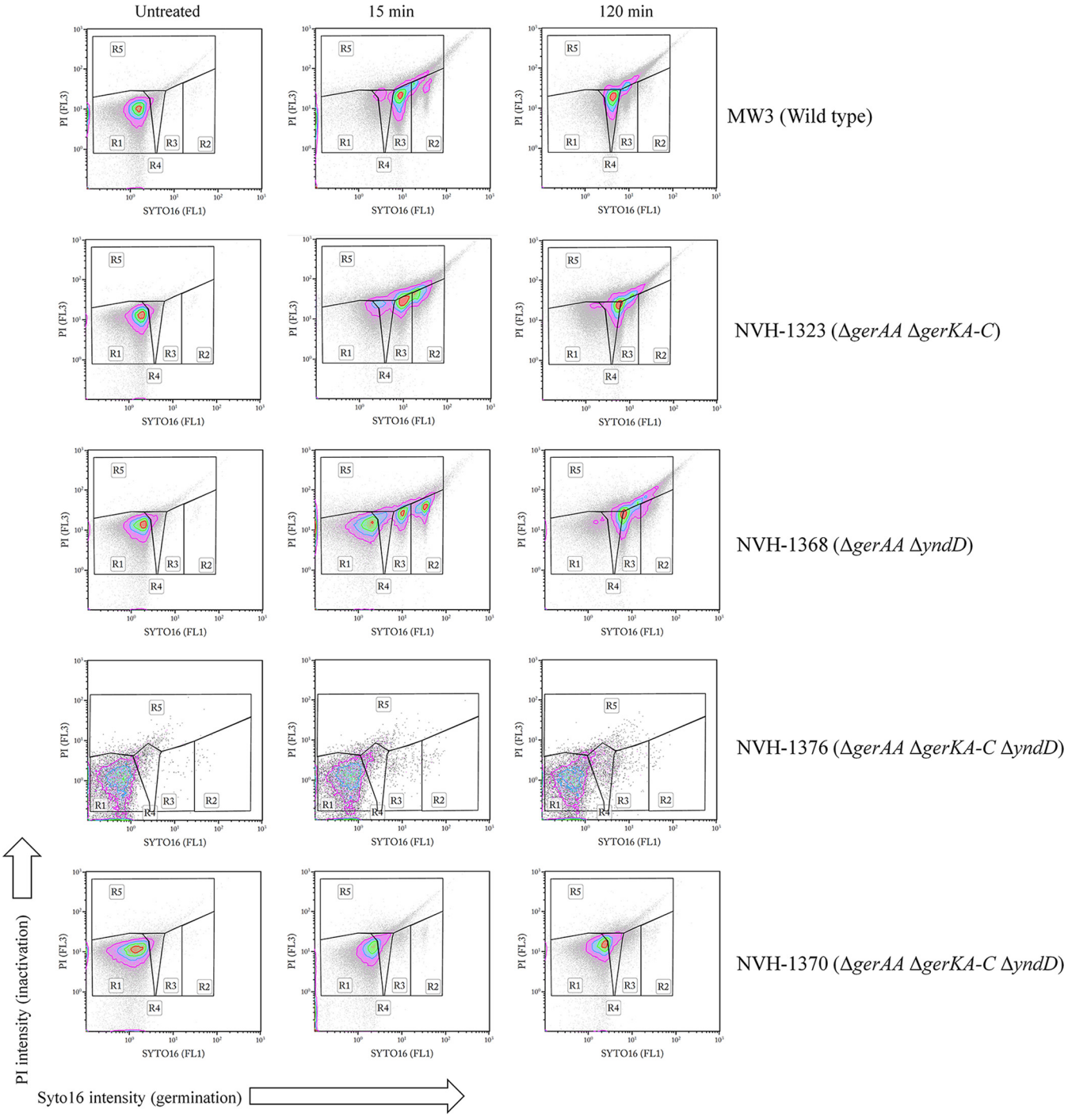
Page 3, Table 1, column 2, row 15: "Δ*yndD* Δ*gerKA-C* mutant" should "Δ*gerAA* Δ*yndD* Δ*gerKA-C* mutant."

Page 5, legend to Fig. 3: "(C) NVH-1376 (Δ*gerKA-C* Δ*yndD*)" should read "(C) NVH-1376 (Δ*gerAA* Δ*yndD* Δ*gerKA-C*)."

Page 5: Lines 19–22 should read as follows. ". . .For spores of strain NVH-1370 (Δ*gerAA* Δ*gerKA-C* Δ*yndD*), which lack all functional GRs (Ger$^-$), no mHP-induced germination was detected, as determined by the plate counts (Table 1 and Fig. 3D)."

Page 6, legend to Fig. 4, line 4: "NVH-1376 (Δ*gerKA-C* Δ*yndD*)" should read "NVH-1376 (Δ*gerAA*::*spc* Δ*yndD* Δ*gerKA-C*)."

Page 6, Fig. 4: "NVH-1376 (Δ*gerKA-C* Δ*yndD*)" should read "NVH-1376 (Δ*gerAA* Δ*gerKA-C* Δ*yndD*)." Figure 4 should appear as shown below.



Page 6, line 1: "(GerA$^+$)" should read "(Ger$^-$)."

Page 6, line 4: "(GerA$^+$)" should read "(Ger$^-$)."

Page 8, lines 31–34: The sentence beginning with "The importance. . ." should be deleted.

Page 11, Table 2, column 2, row 10: "*B. licheniformis* MW3 Δ*gerKA-C* Δ*yndD*" should read "*B. licheniformis* MW3 Δ*gerAA*::*spc* Δ*yndD* Δ*gerKA-C*."

Supplemental material: In Table S1, column 1, "NVH-1376 (Δ*gerKA-C* Δ*yndD*)" should read "NVH-1376 (Δ*gerAA* Δ*gerKA-C* Δ*yndD*)." Revised supplemental material is posted at http://aem.asm.org/content/83/14/e00503-17/suppl/DCSupplemental.