Analysis of Germination Capacity and Germinant Receptor (Sub)clusters of Genome-Sequenced *Bacillus cereus* Environmental Isolates and Model Strains

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ABSTRACT Spore germination of 17 *Bacillus cereus* food isolates and reference strains was evaluated using flow cytometry analysis in combination with fluorescent staining at a single-spore level. This approach allowed for rapid collection of germination data under more than 20 conditions, including heat activation of spores, germination in complex media (brain heart infusion [BHI] and tryptone soy broth [TSB]), and exposure to saturating concentrations of single amino acids and the combination of alanine and inosine. Whole-genome sequence comparison revealed a total of 11 clusters of operons encoding germinant receptors (GRs): GerK, GerI, and GerL were present in all strains, whereas GerR, GerS, GerG, GerQ, GerX, GerF, GerW, and GerZ (sub)clusters showed a more diverse presence/absence in different strains. The spores of tested strains displayed high diversity with regard to their sensitivity and responsiveness to selected germinants and heat activation. The two laboratory strains, *B. cereus* ATCC 14579 and ATCC 10987, and 11 food isolates showed a good germination response under a range of conditions, whereas four other strains (*B. cereus* B4085, B4086, B4116, and B4153) belonging to phylogenetic group IIIA showed a very weak germination response even in BHI and TSB media. Germination responses could not be linked to specific (combinations of) GRs, but it was noted that the four group IIIA strains contained pseudogenes or variants of subunit C in their *gerL* cluster. Additionally, two of those strains (B4086 and B4153) carried pseudogenes in the *gerK* and *gerR*₁ (sub)clusters that possibly affected the functionality of these GRs.

IMPORTANCE Germination of bacterial spores is a critical step before vegetative growth can resume. Food products may contain nutrient germinants that trigger germination and outgrowth of *Bacillus* species spores, possibly leading to food spoilage or foodborne illness. Prediction of spore germination behavior is, however, very challenging, especially for spores of natural isolates that tend to show more diverse germination responses than laboratory strains. The approach used has provided information on the genetic diversity in GRs and corresponding subclusters encoded by *B. cereus* strains, as well as their germination behavior and possible associations with GRs, and it provides a basis for further extension of knowledge on the role of GRs in *B. cereus* (group member) ecology and transmission to the host.

KEYWORDS *Bacillus cereus*, germination, sporeformer, spores

Received 10 September 2016 Accepted 17 November 2016

Accepted manuscript posted online 23 November 2016

Citation Warda AK, Xiao Y, Boekhorst J, Wells-Bennik MHJ, Nierop Groot MN, Abee T. 2017. Analysis of germination capacity and germinant receptor (sub)clusters of genome-sequenced *Bacillus cereus* environmental isolates and model strains. Appl Environ Microbiol 83:e02490-16. <https://doi.org/10.1128/AEM.02490-16>.

Editor Donald W. Schaffner, Rutgers, The State University of New Jersey

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Dormant bacterial spores are able to monitor the environment for conditions that favor growth, indicated by the presence of specific nutrients, such as amino acids, which can trigger spore germination. Germination is a relatively fast biophysical process required to resume vegetative growth that can be initiated by activation of the germinant receptors (GRs) located in the inner membrane of the spore (1, 2). The majority of sporeformers, with *Clostridium difficile* being one of the exceptions (3, 4), contain at least one and usually several GRs that may differ in their specificities for different nutrients (5, 6). The model sporeformer *Bacillus subtilis* 168 carries three functional GRs, of which GerA responds specifically to L-alanine, whereas GerB and GerK cooperate to respond to the mixture of asparagine, glucose, fructose, and K⁺ (AGFK) (1). Notably, spores of the toxin-producing foodborne human pathogen *Bacillus cereus* ATCC 14579 germinate most efficiently in response to a mixture of alanine and inosine and are equipped with seven GRs (GerG, GerI, GerK, GerL, GerQ, GerR, and GerS) that show limited similarity to the GRs present in *B. subtilis* (7). *B. cereus* ATCC 14579 GerR plays a dominant role in germination, as its disruption affected germination in response to many amino acids, purine ribosides, and food products (7, 8). GerG appears to be specifically required for germination with glutamine (7). Additionally, it has been suggested that GRs can respond to more than one germinant and that cooperation of multiple GRs could enhance the germination response with specific individual germinants (6, 9–12). Despite the attempts of Ross and Abel-Santos (13) to standardize the nomenclature used for the GRs, the annotation and naming of GRs are inconsistent across and within spore-forming species; this complicates comparative analysis and prediction of GR specificity.

GRs are usually composed of three subunits (A, B, and C), and genes encoding these subunits are typically arranged in tricistronic operons (14, 15), with some exceptions more frequently found in anaerobic strains of the genus *Clostridium*. The spore GR subunits A and B are integral membrane proteins composed of 5 to 8 and 10 to 12 predicted membrane-spanning domains, respectively (6, 12). Subunit B belongs to a subfamily of single-component membrane transporters and is speculated to be involved in germinant recognition (6). On the other hand, subunit C is membrane associated and conceivably bound to the A and B subunits (6). So far, the exact functions of the individual GR subunits and their interactions have not been elucidated (12). GRs are thought to cluster in complexes, so-called germinosomes, involving the GerD lipoprotein that influences GR-dependent germination rates (16, 17) and possibly the SpoVA channels (located in the spore inner membrane) that are involved in the release of small molecules, mainly dipicolinic acid (DPA) and monovalent cations, and uptake of water during germination (12). Recently, it was shown that heat resistance and the germination rate of *B. subtilis* spores could be attributed to the number of *spoVA*^{2mob} copies on the genome, with a higher number of copies (up to three) correlating with increased heat resistance and reduced germination rate (18, 19).

The spore germination process follows well-described sequential steps (11, 12, 20). Spore swelling of germinating spores and full rehydration of the core require hydrolysis of the peptidoglycan cortex layer. In *B. subtilis*, two germination-specific cortex-lytic enzymes, namely, CwlJ and SleB, are responsible for cortex peptidoglycan degradation (1, 15). After full rehydration, metabolic activity is regained and spore outgrowth is initiated, followed by vegetative growth. Heat activation is commonly applied to enhance fast and homogeneous spore germination; however, the processes involved remain unknown. Spore germination can also be initiated by nonnutrient germinants, including chemical triggers (Ca-DPA and the cationic surfactant dodecylamine), mechanical triggers (high hydrostatic pressure), enzymatic treatment (lysozyme), or bacterial cell wall fragments (muropeptides). These nonnutrient triggers usually bypass GRs and either directly target the release of ions and Ca-DPA or activate cortex-lytic enzymes (1, 2).

Traditionally, germination is monitored by measurement of the optical density at 600 nm (OD₆₀₀) of spore suspensions, with the percent decrease in OD₆₀₀ correlating with germination efficacy assessed by phase-contrast microscopy and/or plate counts

TABLE 1 Germination of heat-activated *B. cereus* spores of 15 food isolates and two laboratory strains exposed to either single amino acids, their mixtures, or complex medium^a

Phylogenetic group	Strain	Heat activated (10 min at 80°C)										
		AlaI no	BHI	TSB	I no	Ala	Cys	Glu	Gly	Val	I so	Av. Blank
II	B4088	98.6	85.5	97.7	76.6	95.6	95.1	14.0	51.8	22.8	19.2	17.1
	B4082	97.5	99.2	97.9	96.0	81.0	56.8	72.4	1.7	6.9	3.0	2.5
	B4147	95.2	97.8	27.6	88.6	90.1	42.5	2.6	1.1	56.5	43.0	1.1
III A	B4085	81.8	13.9	4.1	33.3	1.2	3.8	2.2	3.4	2.2	2.0	1.5
	B4116	71.2	5.0	8.3	37.3	3.5	11.2	9.5	12.1	12.0	8.5	7.9
	B4153	22.2	3.8	4.4	13.2	4.8	4.4	3.4	3.7	4.4	4.1	6.9
	B4086	83.7	50.9	46.5	43.7	9.0	13.7	37.4	10.7	11.5	11.1	11.5
	B4078	98.8	99.8	98.9	99.3	99.5	83.6	32.5	59.6	49.0	34.4	14.7
	ATCC 10987	96.5	95.6	98.4	96.9	97.6	98.5	95.7	77.7	97.1	98.4	36.8
III B	B4087	95.3	89.7	82.3	85.9	51.7	62.6	61.8	63.3	64.3	70.2	54.3
IV	B4084	97.4	99.6	98.9	97.3	94.0	60.5	41.2	3.0	7.4	2.9	4.8
	B4080	98.4	98.7	98.4	14.9	74.7	25.6	13.8	2.9	4.0	2.7	1.4
	B4118	92.4	92.0	96.6	55.5	40.6	15.6	5.6	0.9	5.1	2.8	2.4
	B4155	98.3	98.7	75.5	92.9	95.4	90.0	15.2	50.3	6.3	4.9	3.5
	ATCC 14579	93.0	95.9	98.6	92.6	93.2	94.6	84.3	58.5	90.4	84.1	56.5
	B4158	78.1	80.8	80.1	72.7	78.7	0.0	0.1	0.1	0.1	0.0	6.7
VI	B4117	97.3	95.2	97.4	96.6	87.1	18.1	13.6	16.3	16.1	18.1	16.2

^aSpores showing <15% germination are indicated in red, 15 to 50% germination is in orange, and >50% germination is indicated in green.

(7, 21). Additional methods include measurement of Ca-DPA content by Raman spectroscopy, sometimes combined with laser tweezers (22–24), automated phase-contrast or differential interference contrast (DIC) microscopy, and time-lapse microscopy (for a review, see the paper by Wells-Bennik et al. [20]). Alternatively, spore-staining approaches can be applied in combination with high-throughput analysis of individual spores using flow cytometry (FCM) (25–27). Germinated spores, but not dormant spores, can be stained by DNA fluorescent dyes, such as SYTO-9, since spores lose their structural integrity upon germination, which allows for access of the dye into the spore core and subsequent binding to DNA (25–28).

In the current study, we applied FCM combined with SYTO-9 fluorescent dye staining of germinated spores to evaluate the germination responses of 15 whole-genome-sequenced *B. cereus* food isolates and of two well-studied sequenced laboratory strains, *B. cereus* ATCC 14579 and ATCC 10987 (29, 30), and correlated the germination responses with the presence/absence of Ger clusters in the corresponding genomes. The approach used provided information on the genetic diversity in GRs and corresponding subclusters encoded by *B. cereus* strains, as well as their germination behavior and possible associations with GRs.

RESULTS AND DISCUSSION

Nutrient-induced germination of *B. cereus* spores. The germination behavior of heat-activated spores from *B. cereus* food isolates and laboratory strains was evaluated at the single-spore level under 20 conditions representing saturating concentrations of single amino acids and mixtures of germinants that can be found in food matrices (Table 1; see also Table S2 in the supplemental material).

With the exception of strain B4153, heat-activated spores of all strains showed good germination in response to a mixture of alanine (Ala) and inosine (I no) (i.e., AlaI no). Brain heart infusion (BHI) and tryptone soy broth (TSB) supported germination for most strains. However, a subset of strains belonging to phylogenetic group IIIA (B4085, B4116, B4153, and B4086) showed poor germination in those tested media (BHI and TSB) and in response to individual amino acids. Also, non-heat-activated spores of these strains showed a poor germination response to AlaI no, next to that of B4080 and B4087 (Table S2).

The data presented in Table 1 clearly show diversity in the sensitivity and responsiveness to single amino acids and complex media. We previously showed that the strains used in this study are representative of the diversity found among *B. cereus* strains with respect to carbohydrate utilization and capacity to occupy different environmental niches (soil, food products, and intestinal tract) (31). The poorly germinating group IIIA strains (B4085, B4086, B4116, and B4153) were previously shown to lack specific carbohydrate utilization clusters (those for starch, glycogen, and the aryl beta-glucosides salicin, arbutin, and esculin), suggesting a reduced capacity to utilize plant-associated carbohydrates for growth (31). Since *B. cereus* subgroup IIIA representatives are able to utilize host-associated carbohydrates and carry corresponding carbohydrate utilization gene clusters (31) and a subset of unique Ger (sub)clusters, additional studies using host-derived compounds as germinants may provide further insights into the germination efficacy of their spores.

Notably, all *B. cereus* strains tested carry *spoVA*¹ and *spoVA*² operons (18). Interestingly, only two members of the poorly germinating group IIIA strains, namely, B4085 and B4116, carry a *spoVA*^{2mob} operon, the occurrence of which was recently shown to correlate with slow germination and increased heat resistance of *B. subtilis* spores (18, 19).

Without heat activation, exposure to Ala resulted in very efficient germination of spores of strains B4078 and B4088 and less-efficient germination of spores of strains B4155 and B4158. A study by Broussolle et al. (32) showed significant germination (measured by OD₆₀₀ drop) of spores of a laboratory strain, i.e., *B. cereus* ATCC 14579, upon exposure to 1 mM Ala, while wild *B. cereus* isolates typically required concentrations above 1 mM and, for some, even 200 mM did not result in a maximal OD₆₀₀ drop (32). When the maximum germination rate was reached, a further increase in germinant concentration did not improve germination. Both the maximum germination rate and the minimal concentration to reach this were shown to be strain and germinant dependent (32). Notably, the L-alanine-induced germination response may be affected by the presence of alanine racemase (encoded by the *alr* gene) that is able to convert germination-stimulating L-alanine into germination-inhibiting D-alanine that competitively binds to L-alanine GRs (33, 34). Differences in the L-alanine response (and in the combination with inosine) between strains may be due to differences in Alr activities in the spore cortex.

Cysteine (Cys) has been reported as a potent germinant in *B. cereus* strains (7, 35), and significant Cys-induced germination was indeed observed for heat-activated spores of the majority of the strains tested. Despite the heat activation step, the spores of seven strains (B4085, B4086, B4116, B4117, B4118, B4153, and B4158) stayed insensitive or responded only weakly to one of six individual amino acids: Ala, Cys, glutamine (Glu), glycine (Gly), valine (Val), and isoleucine (Ile). All other strains tested showed enhanced spore germination in response to two or more amino acids tested. The applied 20 or 100 mM concentration of individual amino acids (Ala, Val, Glu, Iso, Cys, and Gly) and Ino used in this study is unlikely to be encountered in the environment; however, it allows fast germination responses. The poorest germination was observed in response to Val and Iso, suggesting that despite an approximately 10 times lower concentration of free amino acids in BHI and TSB (Table S1), their broader composition (Table S1) most likely resulted in a cumulative germination response.

Linking presence of specific GRs to spore germination (genotype to phenotype). GRs are responsible for recognition and binding of the nutrient germinants; however, the exact mechanism in which GR subunits interact and proceed upon germinant binding to the downstream germination pathway remains to be elucidated. Recognizing the fact that annotation of GRs is not a trivial issue and is not consistent across species and within species (6, 13), we present an overview of known GRs and their putative germinant specificity for nine sequenced and well-studied *B. cereus* group reference strains, shown in Table 2.

To compare and assign GRs present in the food isolates to the GRs present in well-studied strains, phylogenetic trees were composed for each GR's A, B, and C

TABLE 2 GRs present in nine selected reference strains

Cluster (this study)	Subcluster (this study)	Putative germinant(s)	Organism	GR name (NCBI) ^a				Cluster ^b	GR name (literature) ^c	Reference(s) ^{c,d}
				Subunit A	Subunit B	Subunit C				
Cluster K		<i>Bacillus anthracis</i> : Pro, Met	<i>B. cereus</i> ATCC 14579	gerKA	gerKB	gerKC		S2	gerK	7
			<i>B. cereus</i> ATCC 10987	gerKA, pseudo	gerKB	gerKC				
			<i>B. cereus</i> E33L	gerKA	gerKB	gerKC				
			<i>B. cereus</i> AH187	gerKA	gerKB, pseudo	gerKC				
			<i>B. thuringiensis</i> serovar konkukian 97-27	gerKA	gerKB	gerKC				
			<i>B. thuringiensis</i> AI Hakam	gerKA	gerKB	gerKC				
Cluster L		<i>Bacillus cereus</i> : Caco-2; <i>Bacillus anthracis</i> : Ser, Val	<i>B. welneshthephanensis</i> KBAB4	gerKA	SGP	SGP		L	gerK	36
			<i>B. anthracis</i> Ames	gerKA	gerKB	gerKC				
			<i>B. cereus</i> ATCC 14579	gerLA, pseudo	gerLB	gerLC				
			<i>B. cereus</i> ATCC 10987	gerLA	gerLB	gerLC				
			<i>B. cereus</i> E33L	gerLA	gerLB	gerLC				
			<i>B. thuringiensis</i> serovar konkukian 97-27	gerLA	gerLB	gerLC				
Cluster G (G _I)		L-Glutamine-mediated germination, L-glutamine plus inosine	<i>B. thuringiensis</i> AI Hakam	gerLA	gerLB	gerLC		A2	gerL	36
			<i>B. welneshthephanensis</i> KBAB4	SGP	SGP	SGP				
			<i>B. anthracis</i> Ames	gerLA	gerLB	gerLC				
			<i>B. cereus</i> ATCC 14579	Pseudo	gerBB	gerBC				
			<i>B. cereus</i> E33L	gerAA	gerBB	gerBC				
			<i>B. cereus</i> AH187	gerAA	SGP	gerAC				
Cluster S		<i>Bacillus anthracis</i> : aromatic amino acids	<i>B. thuringiensis</i> serovar konkukian 97-27	gerSA	gerSB	gerSC		S	gerS	7
			<i>B. cereus</i> ATCC 10987	Pseudo	gerSB	gerSC				
			<i>B. cereus</i> E33L	gerKA	gerLB	gerQC				
			<i>B. cereus</i> AH187	gerSA	gerSB	gerSC				
			<i>B. thuringiensis</i> serovar konkukian 97-27	gerKA	gerLB	gerQC				
			<i>B. thuringiensis</i> AI Hakam	gerKA	gerSB	gerQC				
Cluster X		Virulence, macrophage	<i>B. anthracis</i> Ames	gerSA	gerSB	gerSC		S	gerS2	36
			<i>B. welneshthephanensis</i> KBAB4	SGP	SGP	SGP				
			<i>B. thuringiensis</i> serovar konkukian 97-27	SGP	SGP	SGP				
			<i>B. cereus</i> ATCC 10987	gerXA	gerXB	gerXC				
			<i>B. cereus</i> AH187	SGP	ND	SGP XC				
						SGP LC				
Cluster R		<i>Bacillus cereus</i> : alanine, adenosine, inosine, all amino acids except L-glutamine, food	<i>B. cereus</i> E33L	gerA	gerB	gerAC		Y	gerR	36
			<i>B. thuringiensis</i> serovar konkukian 97-27	gerA	SGP	gerAC				
			<i>B. welneshthephanensis</i> KBAB4	gerA	SGP	SGP				
			<i>B. anthracis</i> Ames	gerYA	Pseudo	gerYC				
			<i>B. cereus</i> ATCC 14579	gerYA	gerB	gerC				
			<i>B. cereus</i> ATCC 10987	gerYA	SGP	gerYC, pseudo				
Cluster Q (Q _I)		Inosine	<i>B. cereus</i> AH187	SGP	SGP	gerAC		Q	gerQ	7
			<i>B. thuringiensis</i> AI Hakam	SGP	SGP	gerAC				
			<i>B. cereus</i> ATCC 14579	gerQA	gerQB	gerQC				
			<i>B. thuringiensis</i> serovar konkukian 97-27	gerQA	gerQB	gerQC				
			<i>B. thuringiensis</i> AI Hakam	gerIA	gerIB	gerIC				
				gerIA	gerIB	gerIC				

(Continued on following page)

TABLE 2 (Continued)

Cluster (this study)	Subcluster (this study)	Putative germinant(s)	Organism	GR name (NCBI) ^a			Cluster ^b	GR name (literature) ^c	Reference(s) ^{c,d}
				Subunit A	Subunit B	Subunit C			
Cluster I		Inosine, L-phenylalanine plus inosine, L-glutamine plus inosine, purine riboside, aromatic amino acid, Caco-2	<i>B. cereus</i> ATCC 14579	gerA	gerB	gerC	I	gerI	7, 59
			<i>B. cereus</i> ATCC 10987	gerHA	gerHB	gerHC			
			<i>B. cereus</i> E33L	gerHA	gerHB	gerHC			
			<i>B. cereus</i> AH187	gerHA	gerHB	gerHC			
			<i>B. thuringiensis</i> serovar konkukian 97-27	gerH	gerHB/SGP IB	gerHC			
Cluster F (F _{II}) Cluster W Not clustering		Unknown Unknown Unknown L-Alanine, L-valine AGFK (in combination with gerK), L-asparagine, many amino acids Unknown	<i>B. weihenstephanensis</i> KBAB4	SGP	SGP	gerAC		gerI	36
			<i>B. anthracis</i> Ames	gerHA	gerHB	gerHC			
			<i>B. cereus</i> AH187	gerKA/gerAC	Hypothetical	gerAC			
			<i>B. cereus</i> AH187	SGP	SGP	SGP			
			<i>B. subtilis</i> 168	yndD	yndE	yndF			
			<i>B. subtilis</i> 168	gerAA	gerAB	gerAC			
			<i>B. subtilis</i> 168	gerBA	gerBB	gerBC			
			<i>B. cereus</i> E33L	gerKA	Extra subunit	gerKC			
					B				
				gerKA	gerKB	gerKC			
Cluster W		Glucose as cogerminant Unknown	<i>B. subtilis</i> 168	gerKA	gerKB	gerKC	K	gerK	21
			<i>B. subtilis</i> 168	yfkQ	yfkT	yfkR			
Not clustering							N/C	yfkQTR	1

^aSGP, spore germination protein; ND, not determined.^bClusters according to nomenclature of Ross and Abel-Santos (13). N/C not clustering.^cThe data in this column refer only to the specific organism in the same corresponding row (for the rows that are blank, there are no corresponding data).^dReferences 1, 7, 8, 21, 36, 59, and 60 and references therein.

subunits of the reference strains and *B. cereus* food isolates (Fig. S2 to S4). Based on these trees, we have identified consistent clustering of corresponding subunits A, B, and C of given types of GRs, illustrating their coevolution, as has been observed previously (2, 13). The clusters were named, when possible, after the GR name/letter of the *B. cereus* type strain, ATCC 14579 (Table 2). The GRs of 29 strains could be allocated to 11 main clusters and their subclusters (Table 3).

B. cereus strains typically harbor a relatively high number of GRs compared with other spore-forming species. Until now, *B. cereus* strains were believed to encompass a core group of five GRs, namely, GerR, GerL, GerK, GerS, and GerI, plus a selection of five additional GRs (6, 36). Indeed, an analysis of 29 genomes revealed that all strains contain *ger* genes belonging to clusters K, I, L, R, and S, albeit that clusters R and S can be differentiated into subclusters R_I and R_{II} and subclusters S_I, S_{II}, and S_{III}, respectively, and that some strains contained one or more pseudogenes encoding putative individual Ger subunits, conceivably resulting in altered functionality of the respective GRs (Table 3). The majority of pseudogenes and/or variants in *gerK* and *gerL* are found in strains belonging to phylogenetic group IIIA, which contains mainly poorly germinating strains. In fact, within both clusters K and L, pseudogenes and/or variants were identified in the genomes of strains B4086 and B4153, which are two poorly germinating strains (Tables S2 and S3). At the same time, two other strains, B4085 and B4116, also belonging to the phylogenetic group IIIA and producing poorly germinating spores (Table S2), carried a variant of the cluster L (subunit C) in combination with GerX_{III}. The combination of those two GRs that are possibly nonfunctional variants could have a negative effect on the germination ability. Both variants of GerL and GerX_{III} are also present in *B. cereus* AH187; spores of this strain were not included in the current germination assays but were previously shown to germinate poorly in response to amino acids or food (36).

Interestingly, GerR, which is a dominant GR in *B. cereus* ATCC 14579 that responds to most amino acids and to model foods (7, 8), belongs to subcluster GerR_I. Differentiation between subclusters GerR_I and GerR_{II} might also reflect functional differences between the two subclusters. In fact, comparative analysis of GerY of *B. anthracis* with unknown function (10) located this Ger receptor in cluster R_{II}, although its subunit C is encoded by a pseudogene and might be a nonfunctional version of the GR variant (Table 3). Moreover, genes encoding subunits C of GerR_I of strains B4086 and B4153, which produce poorly germinating spores, are pseudogenes. Complementation experiments could provide insights into the role of *gerR* in spore germination of other strains. Despite the differences in GR presence/absence, the organization of the GR operons was conserved within the clusters with dominating ABC order, while three clusters (R, F, and Z) followed an ACB organization, clusters X_I and X_{II} revealed a BAC organization, and cluster X_{III} had a unique ACC organization.

In six of the *B. cereus* food isolates and in *B. cereus* AH187, a tricistronic *ger* operon composed of one truncated subunit A and two subunits C was found, creating cluster X_{III}. In *B. subtilis*, the incorporation of subunit C of GerA into the membrane was shown to depend on subunits A (37) and B (38). This suggests either that a different mechanism to form stable receptor assembly (e.g., interaction with subunits of other GRs in the germinosome) is in place in *B. cereus* or that the GR is not functional. Furthermore, subunit B was previously suggested to play a role in nutrient recognition and specificity of the germination response in *Bacillus megaterium* (39, 40). However, the assembly and function of GRs within cluster X_{III} remain to be elucidated.

Spores of strain B4117 (*B. mycoides*) germinated well, even without heat activation, in response to Alano and Ino alone. Strain B4117 encodes two unique (among tested strains) GRs representing clusters Q_{II} and S_{III}. GerQ is known to be involved in Ino-induced germination in *B. cereus* (7), and it could be speculated that the GerQ_{II} version present in B4117 is more responsive than more commonly encountered GerQ_I. Besides B4117, only *Bacillus weihenstephanensis* KBAB4 carries a GR belonging to cluster S_{III}. In fact, both strains also carry GRs from cluster S_{II}, which has been previously referred to as GerS2 (36), while most of the GRs within cluster S belonged to cluster S_I. Notably, van

TABLE 3 Assignment of GRs of selected strains to the clusters identified based on phylogenetic trees of subunits A, B, and C^a

Phylogenetic group	Strain NID/ATCC code ^b	Cluster K	Cluster I	Cluster L	Cluster R		Cluster S			Cluster G			Cluster Q			Cluster X			Cluster F		Cluster W	Cluster Z	NON CLUSTERING
					R ₁	R ₂	S ₁	S ₂	S ₃	G ₁	G ₂	G ₃	Q ₁	Q ₂	Q ₃	X ₁	X ₂	X ₃	F ₁	F ₂			
II	B4088	B - pseudogene																			B - pseudogene		
	B4082																						
	B4077						B - pseudogene																
	B4147																						
	B4079			C - pseudogene																	B - pseudogene		
III A	Bc H4387	B - pseudogene		C variant / pseudogene																			
	B4085			C variant / pseudogene																			
	B4116			C variant / pseudogene																			
	B4153	B - pseudogene		C variant / pseudogene																			
	B4086	C - pseudogene		C - pseudogene																			
	B4078			B - pseudogene, C variant / pseudogene																			
	ATCC10987	A - pseudogene					A - pseudogene																
	Bt Ames					B - pseudogene																	
III B	Bt cerealis Netherlands 97_27																						
	Bt EE3L																						4 component GR
	B4087																						
	Bt At H434m																						
	B4084																						
IV	B4080																						
	B4081																						
	B4118																						
	B4120																						
	B4155																						
	ATCC14579			A - pseudogene																			
	B4158						B - pseudogene																
	B4083								AOK, BAC fragments (poor assembly)														
VI	B4117																						
	Bt H4346																						

^aGreen, presence of GR belonging to given cluster; white, absence of GR belonging to given cluster; gray, at least one of the subunits is a pseudogene or a variant.
^bBa, *Bacillus anthracis*; Bc, *Bacillus cereus*; Bt, *Bacillus thuringiensis*; Bw, *Bacillus weihenstephanensis*.

der Voort et al. (36) previously showed that germination of *B. weihenstephanensis* KBAB4 spores with combinations of selected amino acids and inosine was far more efficient than that of spores from tested *B. cereus* ATCC 14579 and ATCC 10987 strains.

Our study identified additional putative GRs, a group of GRs creating cluster W, including a presumptive *gerT* gene of *B. cereus* AH187 (36), and a putative GR found only in B4079 comprising the one-item cluster Z. Those putative GRs share the tricistronic architecture and homology with known GRs; however, their functionality would require the testing of directed deletion mutants.

Interestingly, the presence/absence of GRs seems to be related to the phylogenetic clustering we reported previously (31). All strains encoding GRs belonging to cluster G_{II} represented phylogenetic group II (Table 3). This group is also characterized by high prevalence (75%) of GRs from clusters S_{II} and Q_I. A phylogenetic tree based on core genes of tested strains (reported by Warda et al. [31]) and phenotypic differences among the strains of group III suggest that two subgroups can be distinguished as IIIA and IIIB. In fact, the majority of strains belonging to group IIIA (and only those) encode GRs from cluster F_{II}. The high prevalence of F_{II} might be compensating for the lack of GRs from cluster Q and high numbers of pseudogenes within clusters K and L. Based on the encoded GRs, experimentally tested strains within group IIIA seem more comparable to strains within group IV than IIIB (Table S3), possibly suggesting similar niche requirements. This may also be reflected by the presence of carbohydrate utilization genes that show similarities for members within those groups. All the strains within group IIIB encode GRs from clusters K, I, L, and S_I. GRs from all the clusters can be found only in strains belonging to group IV, indicating the diversity of this group, with half of the strains encoding GRs from cluster F_I (not found in other strains tested). Finally, strains belonging to group VI often encode alternative GRs; in fact, GRs from clusters S_{III} and Q_{II} are present only in this group, while R_{II} and X_I are found only in one other group. Moreover, strains representing group VI do not encode GRs belonging to cluster G or cluster F (Table 3).

Our study further supports a high degree of diversity in GRs and nutrient-induced germination in spores of different strains of *B. cereus*, generating leads for further studies. However, despite the different germination conditions tested, we could not directly link the presence (or absence) of given GRs to the germination responses such that germination behavior could be predicted. This may be due to the requirement for different types or concentrations of certain germinants or combinations of germinants. A number of factors that can affect the GR-dependent germination have been previously discussed (12, 20, 41), including accessibility and the number of GRs or the downstream germination mechanisms. Moreover, genome-based studies may be affected by the quality of draft genomes, e.g., contig length, the presence of GR subunits (i.e., C subunits) on short contigs, and/or location of GRs or subunits (i.e., those in cluster X_{III}) at the contig's edge. Nevertheless, despite those limitations, the approach presented in the current study allows for the inclusion of rapidly increasing numbers of draft genomes.

Previous studies could not link the diversity in spore germination responses with the presence and/or similarity of the GRs, most likely due to the large number of factors affecting GR-dependent germination (42). Similarly, others (A. O. Krawczyk, A. de Jong, J. Omony, S. Holsappel, M. H. Wells-Bennik, R. T. Eijlander, and O. P. Kuipers, unpublished data) found no correlation between poor germination of *B. subtilis* spores with Ala and the sequences of their GerA subunits. However, the authors did show that "modest germination" with AGFK correlated with the presence of several common amino acid substitutions in the subunits of GerB and GerK (A. O. Krawczyk, unpublished data). Similarly, diverse germination responses to Ala of spores of 46 strains of *Bacillus licheniformis*, which is a close relative of *B. subtilis*, could not be linked with the clusters formed by their *gerAB-gerAC* sequences (44). Nevertheless, complementation of a *gerAA* disruption mutant with *gerA* operons of slow- and fast-germinating *B. licheniformis* revealed that differences in *gerA* family operons are partly responsible for the differences in germination efficiency in response to Ala (44). The greater diversity and

TABLE 4 *B. cereus* strains and reference genomes used in the study

Strain	Strain use ^a	Isolation source/origin	Phylogenetic grouping ^b	Assembly WGS accession no. or UID ^c
<i>B. cereus</i> B4078	E, G	Food, unknown	IIIA	LCYJ000000000.1
<i>B. cereus</i> B4080	E, G	Dried onion	IV	LCYK000000000.1
<i>B. cereus</i> B4082	E, G	Asparagus ham sauce	II	LJKA000000000
<i>B. cereus</i> B4084	E, G	Indian rice dish	IV	LJKC000000000
<i>B. cereus</i> B4085	E, G	Asparagus soup	IIIA	LJKD000000000
<i>B. cereus</i> B4086	E, G	Boiled rice	IIIA	LCYL000000000.1
<i>B. cereus</i> B4087	E, G	Pea soup	IIIB	LCYM000000000.1
<i>B. cereus</i> B4088	E, G	Dressing	II	LJKE000000000
<i>B. cereus</i> B4116	E, G	White sauce	IIIA	LJKF000000000
<i>B. cereus</i> B4117 (<i>B. mycoides</i>)	E, G	Commercial pasteurized milk	VI	LJKG000000000
<i>B. cereus</i> B4118	E, G	Ice cream	IV	LJKH000000000
<i>B. cereus</i> B4147	E, G	Cereals, pasta, and pastries	II	LCYN000000000.1
<i>B. cereus</i> B4153	E, G	Dairy products	IIIA	LCYO000000000.1
<i>B. cereus</i> B4155	E, G	Beef salad	IV	LJKJ000000000
<i>B. cereus</i> B4158	E, G	Vegetables	IV	LCYP010000000
<i>B. cereus</i> ATCC 10987	E, G	Cheese spoilage	IIIA	uid57673
<i>B. cereus</i> ATCC 14579	E, G	Unknown; type strain	IV	uid57975
<i>B. cereus</i> B4077	G	Chilled dessert	II	LCYI000000000.1
<i>B. cereus</i> B4079	G	Canned chocolate beverage	IIIA	LJIT000000000
<i>B. cereus</i> B4081	G	Provolone sauce	IV	LJJZ000000000
<i>B. cereus</i> B4083	G	Tortellini con funghi	VI	LJKB000000000
<i>B. cereus</i> B4120	G	Water	IV	LJKI000000000
<i>B. cereus</i> AH187	G	Vomit; emetic outbreak	IIIA	uid58753
<i>B. cereus</i> E33L	G	Dead zebra isolate	IIIB	uid58103
<i>B. subtilis</i> 168	G	Unknown; type strain	NT	uid57675
<i>B. weihenstephanensis</i> KBAB4	G	Soil isolate	VI	uid58315
<i>B. anthracis</i> Ames	G	Texas, cow; plasmids cured	IIIB	uid57909
<i>B. thuringiensis</i> Al Hakam	G	Suspected bioweapons facility	IIIB	uid58795
<i>B. thuringiensis</i> serovar konkukian 97-27	G	Severe human tissue necrosis	IIIB	uid58089

^aE, strain used for experiments; G, strain used for genome comparisons.

^bBased on the study by Guinebretière et al. (58) and data from Warda et al. (31). IIIA and IIIB refer to divisions within group III based on a core-genome tree (31). NT, not tested.

^cWGS, whole-genome sequencing; UID, unique identifier (NCBI).

complexity of GRs and germination responses among *B. cereus* strains and *B. cereus* group strains compared with, for example, *B. subtilis*, create a challenge for future studies.

In conclusion, our comparative genotyping and phenotyping approach showed that four *B. cereus* strains, B4085, B4086, B4116, and B4153, had poor germination in response to many different germinants and that these strains cluster in phylogenetic group IIIA. These IIIA group strains contain either pseudogenes or variants of genes encoding subunit C in their GerL cluster combined with pseudogenes in cluster K and subcluster R₁ (B4086 and B4153) or combined with the presence of a *spoVA*^{2mob} transposon (B4085 and B4116), which leads to high-level heat resistance with concomitant reduced germination responses in *B. subtilis* spores (18, 19). The approach used has provided information on the genetic diversity in GRs and corresponding subclusters encoded by *B. cereus* strains, as well as their germination behavior and possible associations with GRs, and it provides a basis for further extension of knowledge on the role of GRs in *B. cereus* (group member) ecology and transmission to the host.

MATERIALS AND METHODS

Strains used in this study. Two laboratory strains, *B. cereus* ATCC 14579 and *B. cereus* ATCC 10987, were obtained from the American Type Culture Collection (ATCC) and the culture collection of the Laboratory of Food Microbiology, respectively. In addition, we used 15 sequenced *B. cereus* strains (45, 46) isolated from food products and the food-processing environment (Table 4). Strain B4117 (GenBank accession no. [LJKG000000000.1](#)) was initially included as a *B. cereus* strain but was recently reclassified by the NCBI as *Bacillus mycoides* based on criteria of average nucleotide identity (ANI) typing (47). Strains were cultured in Bacto brain heart infusion (BHI) broth (Becton Dickinson, France) at 30°C with aeration at 200 rpm.

Sporulation conditions. Spores were prepared on a nutrient-rich chemically defined sporulation medium, designated MSM medium, which was previously described (48). Ten milliliters of sporulation

medium was inoculated with 100 μ l of an overnight-grown preculture in 100-ml flasks and incubated at 30°C with aeration at 200 rpm. When the mid-exponential-growth phase was reached (corresponding to an optical density at 600 nm [OD₆₀₀] of ~0.5 [Novaspec II; Pharmacia Biotech, United Kingdom]), 200 μ l of culture was spread on MSM plates (solidified with 1.5% agarose) and incubated at 30°C within plastic bags to prevent drying. After 7 days, 100 mM chilled phosphate buffer (pH 7.4) containing 0.1% Tween 80 was added to the plates, and spores were scraped off the surface and harvested by a 15-min centrifugation at $3,438 \times g$ and 4°C (5804R; Eppendorf, Germany). Spores were washed in decreasing concentrations of Tween 80 and prepared for use as described previously (49). A single spore crop per strain was used for all the experiments.

Germination assay. Fifty microliters of a spore suspension containing approximately 10^8 to 10^9 spores/ml in 100 mM phosphate buffer (pH 7.4) with 0.01% Tween 80 (here referred to as suspension buffer) was exposed to germinants by mixing with 50 μ l of concentrated stock solution of either individual or mixed germinants. The final germinant concentrations used were 20 mM for valine (Val), isoleucine (Ile), glutamine (Glu), glycine (Gly), cysteine (Cys), and inosine (Ino) and 100 mM for L-alanine (Ala), and final concentrations were 10 mM Ala and 2 mM Ino for the mixture of Ala and Ino (Alalno). Amino acid stock solutions were prepared in distilled water and filter sterilized. Control experiments were performed using the complex media BHI and tryptone soy broth (TSB; Becton Dickinson, France) and 25 mM HEPES buffer (pH 7.4). Spores were exposed to germinants for 30 min at room temperature, followed by a centrifugation step for 1 min at $13,000 \times g$ to remove the germinant. The resulting spore pellet was resuspended in 100 μ l of water containing 1 μ M SYTO-9 (Invitrogen, The Netherlands) and incubated in the dark for 10 min at room temperature to stain permeabilized spores. Next, the unbound dye was removed by centrifugation, and the resulting spores were resuspended in HEPES buffer to obtain approximately 1,000 events per second after loading to a FACSAria III flow cytometer (BD, USA). Each experimental run included control spores that were not exposed to germinants. For each sample, 10,000 events were evaluated. For heat activation, spores were heated for 10 min at 80°C in a thermal cycler (Verity; Applied Biosystems, Singapore).

Data analysis. Flow cytometer data were analyzed using the data analysis software FlowJo (version X.0.7; LCC, USA); Fig. S1 in the supplemental material contains the flow chart for data analysis. An approach using SYTO-9 staining and FCM for germination evaluation has been previously validated by Cronin and Wilkinson (25). Scatterplots of forward scatter area (FSC-A) versus a green fluorescence intensity (fluorescein isothiocyanate area [FITC-A]) were used to exclude atypical data sets indicating the presence of spore clumps or strain-specific fluorescence, i.e., high autofluorescence due to the presence of DNA on the spore surface. Events with fluorescence intensity lower than the spore autofluorescence (control sample, FITC-A of $<10^1$) and higher than FITC-A of $>10^5$ were excluded from the analysis.

For each strain individually, the maximum fluorescence of dormant spores was determined based on the cutoff between dormant (unstained) and germinating (stained) spores in control samples (not exposed to germinant) and samples exposed to germinant. The control samples showed dormancy rates between 81.8 and 99.4%, while microscopic observations prior to the experiment showed a minimum of 95% dormancy. Spores with higher fluorescence than the maximum value for dormant spores were considered stained/germinated. Next, a percentage of germinated spores in the population was calculated as a percentage of stained spores among all events considered (unstained and stained).

Per strain, the average percentage of germinated spores in the control samples and its standard deviations were calculated based on three independent measurements, with and without a heat activation step. Three categories of germination performance were defined: spores with $<15\%$ germination (poor germination), spores with 15 to 50% germination (intermediate germination), and spores with $>50\%$ germination (good germination).

Free amino acid analysis. The free amino acid content in BHI and TSB (same lot as used in germination experiments) was determined using liquid chromatography as described previously (50).

Genome mining. To investigate whether the presence of GR operons correlated with experimentally tested germinant-induced spore germination, genomes were mined as described previously (31). The analysis included 20 newly sequenced *B. cereus* food isolates (45, 46), with nine *B. cereus* group strains with publically available genome sequences and with experimentally determined germination responses (6, 36, 48). The strains used and their isolation sources are listed in Table 4. To enhance genome comparisons, additional genomes previously used for *B. cereus* genome comparison were used (31). To improve the comparative analysis, all genomes were (re)annotated using RAST (51), and for the resulting annotated genomes, orthologous groups (OGs; i.e., genes that are descended from the same gene in the last common ancestor of the strains studied, putatively sharing similar functionality) were defined using Ortho-MCL (52). The OGs containing sequences of known germination receptor subunits were extracted from the data set, and additional germination receptor subunits were identified manually by keyword searches and inspection of genome context. Multiple-sequence alignment (MSA) files were made with MUSCLE (53), aligning the protein sequences within specific OGs to facilitate the identification of pseudogenes (encoding incomplete proteins) and manual correction of inaccurate autoannotation using Artemis (54) and Jalview (55). Next, phylogenetic trees of individual GR subunit A, B, or C based on the aligned amino acid sequences of the 29 *B. cereus* group strains and *B. subtilis* 168 were constructed using Clustal X (56) and visualized using LOFT (57) (Fig. S2 to S4). When subunits A, B, and C of a given type of GRs clustered consistently in one of the 11 clusters (and their subclusters), the candidate GR was assigned the GR name/letter of the well-studied strain, preferentially *B. cereus* ATCC 14579 (Table 2). Newly identified and known GR operons are summarized in Table 3 using the updated GR naming system (Table 2).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02490-16>.

TEXT S1, PDF file, 2.1 MB.

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