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Influence of Sporulation Medium Composition on Transcription of *ger* Operons and the Germination Response of Spores of *Bacillus cereus* ATCC 14579

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***Bacillus cereus* ATCC 14579 endospores were produced in Y1 medium, a nutrient-rich, chemically defined sporulation medium, and in modified G medium, containing low amounts of nutrients. The average transcription level of the seven *ger* operons per cell was 3.5 times higher in Y1 medium, and the spores grown in this medium showed an enhanced germination response.**

Bacillus cereus is a gram-positive, rod-shaped food pathogen that can sporulate under nutrient-limiting conditions. The conditions under which sporulation takes place determine spore characteristics such as wet-heat resistance and germination properties (7, 18, 22, 26). Once formed, spores can return to vegetative growth by an irreversible process called germination (19, 21, 24). Germination receptors, which are located in the inner membrane of the spore, play a crucial role in the initiation of germination, as spores lacking these receptors are strongly impaired in their responses to germinant molecules (20). The signaling and activation pathway(s) mediated by these receptors is still unknown. It has been shown, however, that spores contain different types of receptors that can respond to specific germinant molecules, mostly amino acids or purine ribonucleosides (1, 4, 10, 11, 13, 19, 27). Germination receptors are encoded by tricistronic operons called *ger* operons, and the three gene products are necessary to form a functional receptor (19). During sporulation, the *ger* operons are transcribed in the forespore by a sigma G-dependent RNA polymerase (21). The genome of *B. cereus* ATCC 14579 contains seven putative *ger* operons, which may equip the spore with a set of seven functional receptors (11). The *Bacillus subtilis* genome contains five *ger* operons, of which three have been characterized (5, 14, 28). These three *ger* operons were expressed at very low levels (5, 8, 28). The composition of the medium can affect various spore properties (3, 7, 9), but the effect of medium composition on the transcription of the *ger* operons and its impact on germination properties of the spores is not known. Changes in *ger* operon expression may cause variation in the number of receptors in the spore, which consequently affects the nutrient-induced germination properties.

This report describes the transcriptional analysis of each of the seven *ger* operons of *B. cereus* ATCC 14579 during sporulation in nutrient-rich Y1 medium, containing approximately 30 mM amino acids and 10 mM glucose, and in modified G medium, containing approximately 14 mM amino acids and no glucose. The composition of the medium had a significant impact on expression of the *B. cereus ger* operons and the spores' nutrient-induced germination characteristics.

Bacterial strains, spore preparation, and transcriptional analysis. The *B. cereus* ATCC 14579 *ger* mutant strains used were disrupted in each of the seven *ger* operons by the insertion of plasmid pMUTIN4 as described previously (11). For all insertions, the *lacZ* reporter gene present on pMUTIN4 was under the control of the *ger* operon promoter, facilitating the measurement of transcriptional activity under different sporulation conditions. Spores of the wild-type and mutant strains were prepared on a nutrient-rich, chemically defined sporulation medium designated Y1 medium, which contained the following components (final concentrations): D-glucose (10 mM), L-glutamic acid (20 mM), L-leucine (6 mM), L-valine (2.6 mM), L-threonine (1.4 mM), L-methionine (0.47 mM), L-histidine (0.32 mM), sodium-DL-lactate (5 mM), acetic acid (1 mM), $FeCl_3$ (50 μM), $CuCl_2$ (2.5 μM), $ZnCl_2$ (12.5 μM), $MnSO_4$ (66 μM), $MgCl_2$ (1 mM), $(NH_4)_2SO_4$ (5 mM), Na_2MoO_4 (2.5 μM), $CoCl_2$ (2.5 μM), and $Ca(NO_3)_2$ (1 mM). The medium was buffered at pH 7.2 with 100 mM potassium phosphate buffer (6). Furthermore, spores were prepared on modified G medium as described previously (15); the medium contained 0.2% yeast extract, $CaCl_2$ (0.17 mM), K_2HPO_4 (2.87 mM), $MgSO_4$ (0.81 mM), $MnSO_4$ (0.24 mM), $ZnCl_2$ (17 μM), $CuSO_4$ (20 μM), $FeCl_3$ (1.8 μM), and $(NH_4)_2SO_4$ (15.5 mM) and was adjusted to a pH of 7.2. This medium was expected to contain approximately 14 mM amino acids, based on a 70% protein content of the yeast extract. Cultures were incubated at 30°C with shaking at 225 rpm, which resulted in >99% free spores in both media, after incubation for 48 h. The spores were then harvested, washed repeatedly, and stored as described previously (11).

Transcriptional activity of the *ger* operons during sporulation was measured by determining the level of β-galactosidase activity using the 4-methylumbelliferyl-β-D-galactoside (MUG) assay. One-milliliter samples of a sporulating wild-type or mutant culture were taken at 5, 10, 15, 20, and 25 h after inoculation and washed and stored at −20°C until

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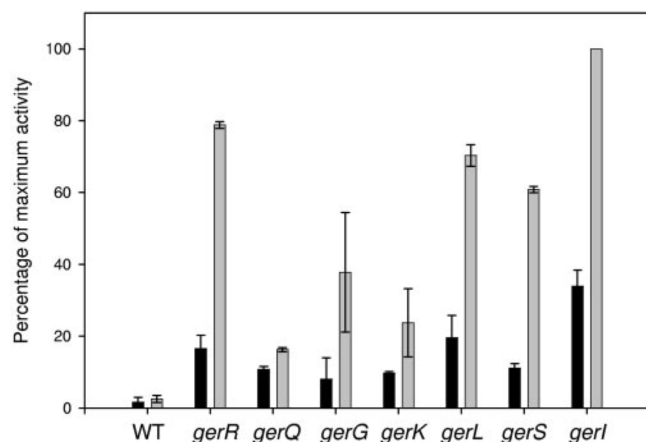

FIG. 1. Relative β-galactosidase activity expressed from the *ger-lacZ* fusions constructed for the seven *ger* operons of *B. cereus* during sporulation on modified G (black bars) and Y1 (gray bars) sporulation media. Maximum β-galactosidase activity was measured 10 h after the onset of sporulation in the respective medium. The number of enzyme units was calculated per ml of culture and corrected for the observed difference in cell density. Data are expressed as a percentage of the maximum value obtained with the *gerI-lacZ* construct (20 pmol MUG hydrolyzed per ml of culture). WT, wild type.

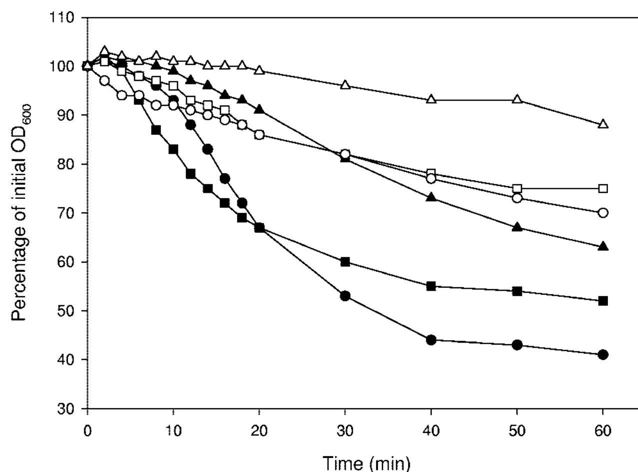

FIG. 2. Germination of *B. cereus* wild-type spores produced on Y1 (closed symbols) and modified G (open symbols) media. Spores were germinated with 1 mM L-alanine (circles), L-cysteine (squares), and L-threonine (triangles).

assayed. The β-galactosidase activity was assayed by measuring the fluorescence that resulted from the conversion of MUG to 4-methylumbelliferone with a Tecan fluorometer as described previously (12). The instrument was calibrated with a 4-methylumbelliferone calibration curve. Spore germination was monitored by measuring the reduction of the optical density at 600 nm (OD_{600}) of the spore suspension as described previously (10). The data presented are the result of three independent experiments.

Growth and sporulation in Y1 and modified G sporulation media. In both media, vegetative growth was observed first before the cells entered sporulation. After 4 h of vegetative growth in modified G medium, at which point a maximum cell density of 1.5 (OD_{600}) was reached, the cells entered sporulation. For Y1 medium, vegetative growth was observed for 12 h, at which point a maximum cell density was reached (OD_{600}, 4.5), and the cells entered sporulation. The *B. cereus* ATCC 14579 wild type and seven *ger* mutants displayed similar growth characteristics, including growth rates, final ODs, and spore yields during sporulation. The shift from growth to sporulation was accompanied by strong aggregation of the cells. Sporulation in the two media was synchronous and complete.

Transcription levels of the *ger* operons in Y1 and modified G sporulation media. Transcription levels of the *ger* operons were determined by measuring the amount of β-galactosidase produced in each of the seven *ger* mutant strains at five time points during sporulation. Transcription of the *ger* operons reached a maximum approximately 10 h after the start of sporulation. At 20 h after the onset of sporulation, the maturing spores became increasingly insensitive to lysozyme treatment. Because the onset of sporulation in the two media started at different time points, a mutual comparison of β-galactosidase levels of samples taken at identical time points was not possible. Therefore, we compared the maximum expression levels of each *ger* operon of sporulating *B. cereus* cells in both media. In Y1 medium, the maximum expression levels of the *ger* operons during sporulation were substantially higher than in modified G medium (Fig. 1). On average, expression of the seven *ger* operons was 3.5 times higher per cell in Y1 medium than in spores produced in modified G medium. Furthermore, differences in expression levels for the single *ger* operons were noticed, although in both media, the highest transcription level was observed for the *gerI* operon encoding a germination receptor involved in purine ribonucleoside-initiated germination (4, 11). Although the mutants harbor the same promoterless *lacZ* gene, diversity in the ribosome binding sites and translation start codons of the *ger* mutants may influence the β-galactosidase levels originating from these constructs. Therefore, we cannot be sure that the relative levels of β-galactosidase are a precise reflection of the relative levels of the Ger receptors. A comparison of the levels of β-galactosidase produced in the two media was done with the same strains, and this reflects a valid comparison of the β-galactosidase levels produced in the two media.

Germination characteristics of spores produced on Y1 and modified G media. To assess the germination characteristics of spores prepared in both media, spore germination assays were performed using the amino acids L-alanine, L-cysteine, and L-threonine and the purine ribonucleosides inosine and adenosine as germinant molecules. For both amino acid- and purine ribonucleoside-induced germination, spores prepared on modified G medium exhibited altered germination characteristics compared to spores produced on Y1 medium. This was demonstrated by reduced germination rates for amino acid-induced germination (Fig. 2) and a reduced germination rate and a more pronounced lag phase for purine nucleoside-induced germination (Fig. 3) for spores grown on modified G medium. Spores from modified G medium required, on average, 10-fold higher concentrations of germinants to accomplish germination rates and efficiencies similar to those of Y1-medium-grown spores (data not shown). By adding a synergistic combination of L-alanine and inosine, resulting in a powerful germinant mixture, spores originating from both media showed

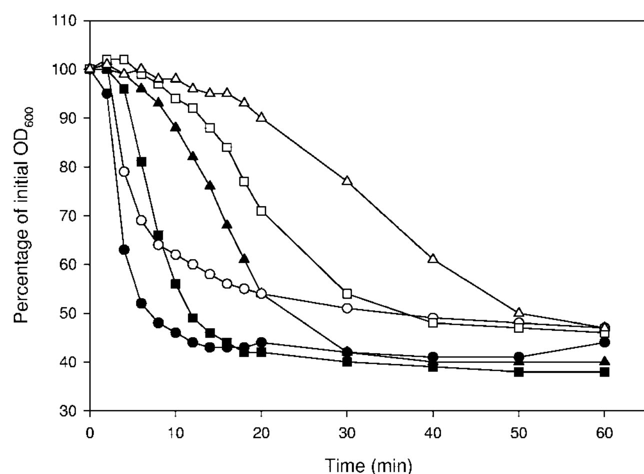

FIG. 3. Germination of *B. cereus* wild-type spores produced on Y1 (closed symbols) and modified G (open symbols) media. Spores were germinated with a combination of 10 mM L-alanine and 1 mM inosine (circles), with 1 mM inosine alone (squares), and with 1 mM adenosine (triangles) alone.

immediate responses and high, almost similar, germination rates (Fig. 3). This indicates that a potent germination trigger may overcome a limited number of nutrient receptors. To analyze whether these differences in spore germination characteristics resulted from an early (stage 1) germination event (24), both types of spores were germinated with Ca^{2+}-dipicolinic acid. This nonnutrient germinant induces germination while bypassing the individual components (i.e., the Ger receptors) of the nutrient germination pathway. Ca^{2+}-dipicolinic acid-induced germination showed equal rates and efficiencies with both types of spores, indicating that the germination pathways after stage 1 are similar in spores originating from Y1 and modified G media (data not shown). This observation points toward a difference in the first stage of germination, and based on the transcriptional analysis of all *B. cereus* ATCC 14579 *ger* operons, it is conceivable that the number of germination receptors is lower in spores prepared on modified G medium, resulting in reduced germination rates and efficiencies.

It has been described previously that the level of *ger* operon transcription can affect germination characteristics. Overexpression of the *gerA* operon in *B. subtilis*, which elevates the level of the GerA proteins, resulted in a more rapid response for L-alanine-induced germination (2). Therefore, the lower number of germination receptors that result from sporulation in modified G medium may result in a diminished germination rate.

For purine ribonucleoside-induced germination (Fig. 3), the low levels of expression of the *ger* operons measured in modified G medium-grown spores correlated with a prolonged lag phase before germination started. Once started, the germination rates and efficiencies were only slightly lower for the modified G medium-produced spores. As discussed previously, the amino acid-induced germination seems to proceed via a partially different pathway than the purine ribonucleoside-induced germination (11, 25). It is known that the integument and exosporium of the spore harbor active enzymes, including inosine hydrolase, that may convert compounds added to the spore suspension, such as purine ribonucleosides, into effective germinants (16, 17, 23). Such enzymes may play an essential role in purine ribonucleoside-induced germination, and the observed lag phase may be caused by the time needed to convert adenosine or inosine into effective concentrations of the actual germinant molecule(s). This resulting molecule may initiate germination mediated by, for example, the GerI receptor, which has been shown to be involved in the inosine and adenosine responses in *B. cereus* ATCC 14579 (1, 11). Assuming that lower numbers of receptors are present in spores originating from modified G medium, a higher concentration of the actual germinant molecule may be required before germination can proceed. Consequently, more time is needed to produce a sufficient quantity of this germinant molecule, resulting in the observed prolonged lag phase. For amino acid-induced germination, the germination rate correlates with the concentration of the germinant (10), and the assumption that spores produced on modified G medium contain a lower number of receptors may be reflected directly in the germination rate.

In the environment, sporulation conditions can be extremely diverse, and properties of the resulting spores, including germination efficiencies, may differ significantly. This large diversity in spore properties may add to the success of spore dispersal in foods and affect spore survival and outgrowth potential after the imposition of food preservation technologies.

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