APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 2004, p. 2514–2519 Vol. 70, No. 4
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.4.2514–2519.2004

Growth and Sporulation of *Bacillus cereus* ATCC 14579 under Defined Conditions: Temporal Expression of Genes for Key Sigma Factors

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Received 4 August 2003/Accepted 26 December 2003

An airlift fermentor system allowing precise regulation of pH and aeration combined with a chemically defined medium was used to study growth and sporulation of *Bacillus cereus* ATCC 14579. Sporulation was complete and synchronous. Expression of *sigA*, *sigB*, *sigF*, and *sigG* was monitored with real-time reverse transcription-PCR, and the pattern qualitatively resembled that of *Bacillus subtilis*. This method allows reproducible production of stable spores, while the synchronous growth and defined conditions are excellently suitable for further gene expression studies of cellular differentiation of *B. cereus*.

Bacillus cereus is a gram-positive, facultative anaerobic rod-shaped endospore-forming bacterium. *B. cereus* occurs ubiquitously in soil and in many raw and processed foods such as rice, milk and dairy products, spices, and vegetables (4, 7, 14, 34, 36). Many strains of *B. cereus* are able to produce toxins and cause distinct types of food poisoning (13, 22). Concerns over *B. cereus* contamination have increased because of the rapidly expanding number of chilled foods that may be pasteurized but may still contain viable spores (4, 14). Spores from *B. cereus* can germinate and outgrow during storage, even at low temperatures (4, 6, 14). Major efforts to battle this increasing problem are focusing on determining the causes of the spore's resistance and the mechanisms of germination.

There is ample literature on spores and sporulation of *B. cereus*, but for most sporulation studies that focus on genetics, derivatives of *Bacillus subtilis* 168 are used, due to their easy handling and genetic accessibility (9). *B. subtilis* has a much smaller genome (21) than that of *B. cereus* and contains no plasmids, while *B. cereus* strains are commonly known to harbor one or more plasmids (15). Genome analysis indicates that *B. cereus* and *B. subtilis* have completely different ecological backgrounds: whereas *B. subtilis* can be seen as a benign soil dweller specialized in degrading plant-derived polysaccharides, *B. cereus* seems to be adapted to thrive as a pathogen or parasite, preferring a more carnivorous diet of proteins and amino acids (17, 29). Differences include important sporulation genes, for example, in *gerA*-type operons, the products of which are essential to nutrient- and nonnutrient-mediated germination of spores (28, 42). The *B. subtilis* genome contains five *gerA*-type operons of which only three are expressed during sporulation (28), while data mining of the *B. cereus* genome has revealed the presence of seven *gerA*-like operons, which are all expressed during sporulation (L. M. Hornstra, Y. P. de Vries, M. H. Bennik, W. M. de Vos, and T. Abee, presented at Functional Genomics of Gram-Positive Microorganisms, 12th International Conference on Bacilli, Baveno, Italy, 22 to 27 June 2003). Furthermore, *B. cereus* spores are surrounded by an exosporium, whereas *B. subtilis* spores are not (20, 39). Thus, although many of the genes that play a role in sporulation are believed to be conserved among bacilli (10, 37), there may be important differences between *B. cereus* and *B. subtilis* sporulation genes and their expression.

It has been well established that bacterial spore properties are affected by the conditions during sporulation (2, 11, 12, 23, 33), but in most studies spores are routinely produced from fortified agar or rich liquid media, which results in heterogeneous sporulation conditions for the individual cells. Homogeneous sporulation conditions and precise regulation of growth and sporulation parameters are of great importance for obtaining reproducible and homogeneous spore batches. Furthermore, the study of gene expression during cell differentiation requires homogeneous and synchronized cultures.

In this study we describe defined conditions for growth and sporulation of *B. cereus* ATCC 14579, which has been characterized at the genome level (17), in a chemically defined medium combined with an airlift fermentor system. We monitored the expression of four genes coding for sigma factors involved in housekeeping, stress response, and sporulation (σ^A, σ^B, σ^F, and σ^G) during growth and sporulation. Important properties of the resulting spores are established and discussed, and the temporal expression of the sigma factor genes is compared to the sporulation model developed for *B. subtilis*.

Strains, medium, and fermentation. *B. cereus* strain ATCC 14579 was obtained from the American Type Culture Collection and grown on a chemically defined medium modified from the work of Nakata (25), 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.

The fermentor system consisted of an autoclavable 2-liter glass bioreactor from Applikon (Schiedam, The Netherlands), equipped with an ADI 1020 biocontroller unit, an ADI 1012

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motor controller, and an ADI 1018 thermocirculator. Sterile air was fed through the culture at a constant rate while the oxygen concentration was kept at 50% saturation level by automatic adjustment of the stirring speed. Fermentations were carried out at a constant temperature of 30°C. The fermentor was inoculated with approximately 4×10^8 *B. cereus* spores that had been pasteurized at 75°C for 15 min and subsequently germinated with a mixture of L-alanine and inosine (25 and 1 mM, respectively) in 10 mM potassium phosphate buffer at pH 8.0 for 5 min at 30°C.

Analytical procedures. Concentrations of D- and L-lactic acid and glucose were determined in culture supernatant by the UV method with enzymatic bioanalysis kits from Boehringer Mannheim (Darmstadt, Germany). Phase-contrast microscopy was performed with an Axioskop microscope from Zeiss, equipped with a Coolpix 995 digital camera from Nikon.

Spore handling and properties. Spores were harvested through airlifting as the spores stabilized the foam formed in the top of the fermentor vessel. The foam containing the spores passed through the fermentor air outlet and was fed through two bottles in which it slowly collapsed. What remained was a highly concentrated suspension containing 90 to 99% phase-bright spores, as monitored by phase-contrast microscopy. This suspension was centrifuged at 10,000 × *g* for 30 min. The spores formed a solid pellet with a light cream color, on top of which in some cases a thin, loosely packed, dark brownish upper layer was present. This upper layer consisted of cell debris and was discarded while the remainder of the pellet was resuspended in sterile water. The spores were kept at room temperature and subjected to daily washes in water until there was no cell debris, vegetative cells, or phase-dark spores left (typically after three to seven washings). Subsequently, the spores were suspended in 10 mM KPO_4 buffer at pH 7 and stored in the dark at 4°C.

The heat resistance assay was modified from reference 19. Spores (10^6 to 10^7/ml) were injected at regular time intervals in screw-cap tubes containing 10 mM KPO_4 (pH 7) at 100°C in an oil bath. After a set time, all tubes were cooled simultaneously on ice. Serial dilutions were made in 10 mM KPO_4 (pH 7) and plated onto diluted nutrient broth (2.6 g/liter; Difco) solidified with 1.5% agar. Colonies were counted after 3 days of incubation at 30°C.

Spore surface hydrophobicity was measured according to the method described in reference 35. Spores were suspended in water, and after measurement of the A_{660} (*A* before; values of 0.4 to 0.5), 0.1 ml of *n*-hexadecane (Sigma Aldrich) was added to 2 ml of spore suspension in a glass tube. This mixture was vortexed for 1 min, after which the phases were allowed to separate for 15 min. Then, the A_{660} of the aqueous phase was determined (*A* after), and the percent transfer to the *n*-hexadecane was deduced by calculating 100 − [(*A* after/*A* before) × 100].

The wet density of the whole spore was measured according to the method in reference 38. Spores were concentrated in 0.15 M NaCl, added to 90% (vol/vol) Percoll (Amersham Pharmacia Biotech) solution with 0.15 M NaCl, and subsequently centrifuged for 20 min at 30,000 × *g*. Whole-spore wet density was derived by comparison of the positions of the spores with those of density marker beads (Amersham Pharmacia Biotech) in the self-established gradient.

RNA isolation and real-time reverse transcription-PCR. For RNA extraction we used the RNeasy kit from Qiagen. Cells from 2 ml of culture were resuspended in 0.6 ml of RLT buffer provided in the RNeasy kit and lysed by bead beating (3 min at 30 Hz) with 0.6 g of zirconia silica beads (0.1-mm diameter) from Biospec (Bartlesville, Okla.). The lysate was centrifuged for 2 min at the highest speed in an Eppendorf centrifuge, and the supernatant was used in the protocol provided with the RNeasy kit. The purified RNA was treated with RNase-free DNase (Amersham Pharmacia Biotech). One hundred twenty nanograms of total RNA of each sample was used for cDNA synthesis. In silico analyses were performed with the ERGO genome and discovery system (27) from Integrated Genomics (Chicago, Ill.). Appropriate primers (all 5′ to 3′) were designed for *sigA* (*sigA*F, CTATGTAGGCCGTTGGTATGCCT; *sigA*R, AGGCGATGTTGCTTCTTGGTCTT), *sigB* (*sigB*F, GAAATCGCAAATCATTTAGG; *sigB*R, CTTTTAATACG AGAAACGTG), *sigF* (*sigF*F, GGATGTATTGGGCTCTTG AAATCGG; *sigF*R, CGGCTCGCTTCTTGTGCTAGAACA AC), and *sigG* (*sigG*F, GCAAAGTGGAGAGATAAGCGCA AGAG; *sigG*R, TACGCGAATCGGATTATTATCACGC). Copy DNA was synthesized with Superscript II (Invitrogen) according to the manufacturer's instructions, in a final volume of 20 μl. For real-time PCR we used the qPCR core kit for Sybr Green I-No ROX, from Eurogentec. Amplification reaction mixtures contained 2 μl of cDNA template and 10 pmol of each primer (R + F) in a final volume of 50 μl. In deviation from the protocol delivered with the qPCR core kit, 0.5 μl of 1,000-fold-diluted Sybr Green I solution from Molecular Probes was used instead of the Sybr Green from the qPCR core kit. The amplification profile was 30 s at 94°C, 30 s at 58°C, and 60 s at 72°C for *sigA*, *sigB*, and *sigF*. For *sigB* the temperature of step 2 was lowered from 58 to 55°C. PCR products were detected directly by monitoring the increase in fluorescence caused by Sybr Green I intercalation with the iCycler system (Bio-Rad Laboratories). A threshold was set, which intersected the amplification curves in the linear region of the semilog plot. The original amounts of cDNA in unknown samples are compared to each other by interpolation from a standard curve of Ct (threshold crossing) values generated from different concentrations (10, 1, 0.1, and 0.01 ng/μl) of genomic DNA, isolated from exponentially growing *B. cereus* cells according to the method in reference 31. The sigma factor expression level was calculated as expression level = ae^{bCt}, *in which a* and *b* are constants dependent on the primer set and amplification parameters, experimentally derived from the standard curve with the genomic DNA as a template. Ct is the threshold crossing value found in the amplification reaction with the specific primer set, reaction parameters, and cDNA from the specific time point as a template. All reactions were carried out in duplicate, and the Ct values of the duplicates differed in all cases less than 5%. All of the amplified fragments used to generate data points had melting curves identical to those of the positive controls and formed single bands of the expected sizes on an agarose gel, confirming that the rise in fluorescence measured by the iCycler system was caused by amplification of only the specific gene fragments that we targeted.

Growth and substrate utilization. To link temporal expression of genes to substrate availability and growth phase, we

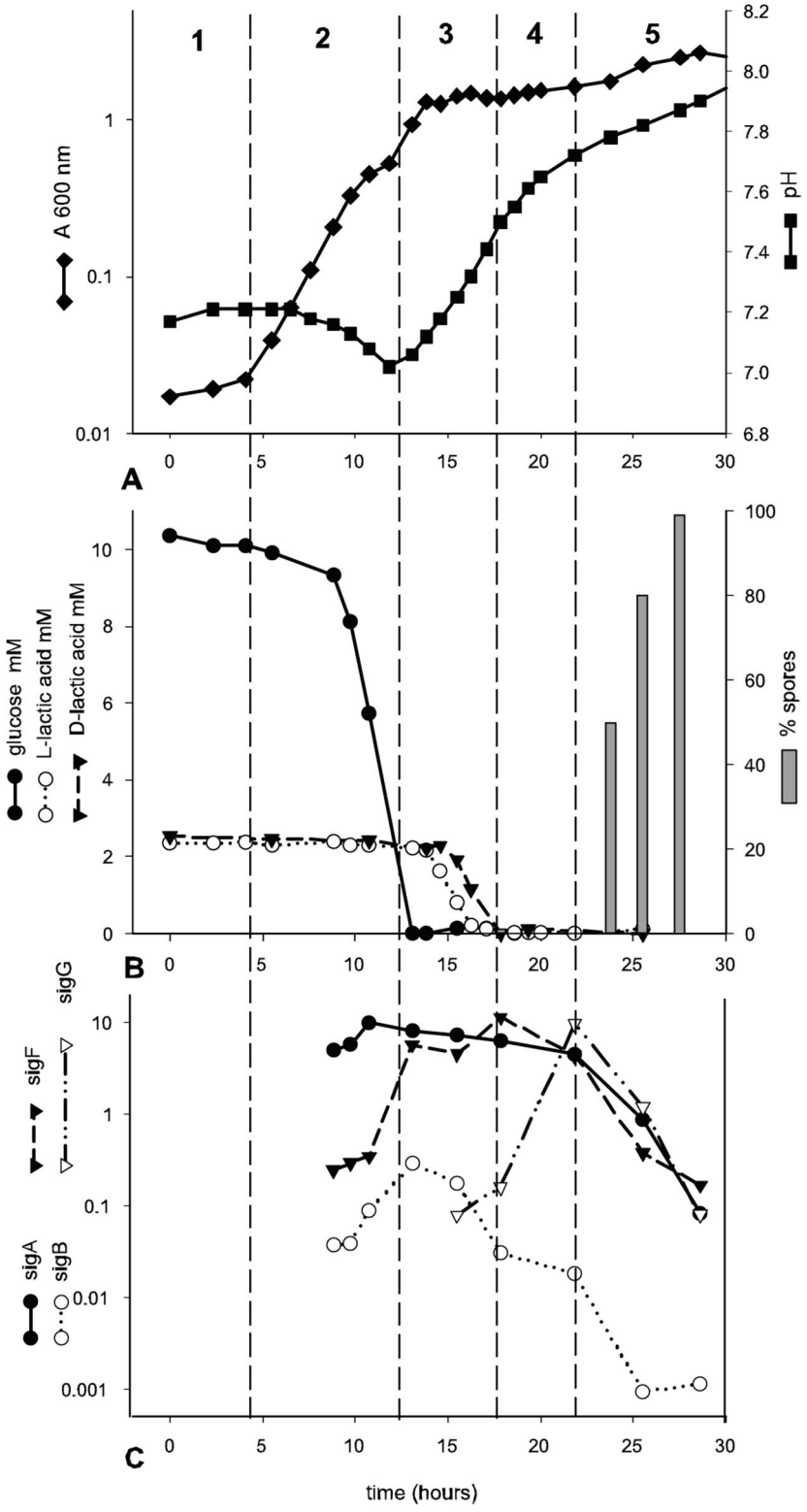

FIG. 1. Growth and sporulation of *B. cereus* in a defined medium. (A) A_{600} and pH; (B) glucose, D-lactic acid, and L-lactic acid millimolar concentrations and the percentages of cells with a phase-bright spore; (C) expression levels (calculated as described in the text) of *sigA*, *sigB*, *sigF*, and *sigG*. The vertical broken lines divide the graphs into five developmental phases: lag (1); exponential (2); early stationary (3); late stationary, early sporulation (4); and late sporulation (5) phases.

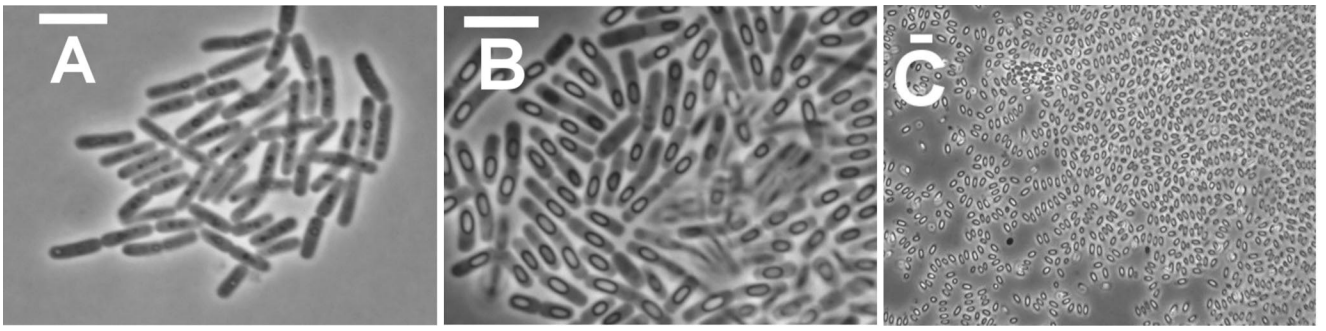

FIG. 2. Microscopic images of *B. cereus* cells. Bars, 5 μm. (A) Vegetative cells aggregating in stationary phase; (B) aggregated cells forming spores; (C) spores present in foam.

began by determining the growth characteristics and the substrate utilization (Fig. 1). The growth and pH development during growth and sporulation were roughly similar to those previously described (18, 24, 26). However, we could clearly distinguish five different growth phases, starting with a lag phase (phase 1). In the exponential phase (phase 2) cells occurred singly or in short chains and were highly motile. During this phase, the pH in the culture dropped from 7.2 to 7.0 but started to rise again as soon as the glucose was depleted (phase 3), indicating that the decrease in pH resulted from fermentation of glucose. Apparently, even with oxygen at a 50% saturation level in the culture medium, the bacteria could not take up oxygen sufficiently fast to support complete glucose respiration. Homolactic acid fermentation of glucose was not likely to occur, because the concentration of lactic acid did not increase. However, *B. cereus* has been reported to ferment glucose to other acids (26). Furthermore, lactic acid is not metabolized by the bacteria as long as glucose is present (phases 1 and 2), probably due to catabolite repression. Upon glucose exhaustion the cells entered stationary phase (phase 3), lost their motility, and became arranged into aggregates (Fig. 2A), while lactic acid was metabolized. Cell aggregation in *B. cereus* has been reported to be a specific event (41) and may play a role in the transfer of genetic material, which occurs frequently within the *B. cereus* group (15). *B. cereus* metabolized both D- and L-isomers of lactic acid with a slight preference for the L isomer (Fig. 1B). Lactic acid metabolism caused a dramatic increase of the pH.

At the end of the late stationary, early sporulation phase (phase 4) the first phase-gray spores could be seen in the aggregated cells. The number of phase-bright spores increased suddenly and dramatically in the final sporulation phase (phase 5). During this final stage of spore formation, the absorbance increased further, as in the end over 99% of the cells had a phase-bright spore (Fig. 1 and 2B). Finally, the aggregates of cells fell apart, after which the cells lysed and the spores were released into the medium. Spore purification was facilitated by the high degree of sporulation, because almost no vegetative cells were left in the spore suspensions. Furthermore, the cells formed spores at the same time, in phase 5, confirming synchronicity in the culture. After release from the sporangia, most of the spores aggregated in large amounts of foam formed in the top of the fermentor and were airlifted out of the vessel. We used this property to harvest the spores without the need to centrifuge all the culture liquid. The foam was collected, and microscopic observation confirmed that it consisted almost exclusively of phase-bright spores (Fig. 2C).

We tested the mature spores for a number of parameters that are important in relation to food processing, such as wet heat resistance and hydrophobicity. The obtained spores were stable over time and showed a high resistance to wet heat, with a reproducible decimal reduction time of 1 min at 100°C, comparable to previous reports (1). Spore surface hydrophobicity is caused by the presence of an exosporium (20) and plays a key role in adherence of the spores to food and food processing equipment surfaces (5, 40). The obtained *B. cereus* spores were highly hydrophobic, since as much as 95% of the spores transferred from the water to the *n*-hexadecane phase (data not shown). In contrast, of *B. cereus* vegetative cells and *B. subtilis* spores more than 98% (data not shown) remained in the water phase, which is consistent with previous reports (20, 40). We measured the whole-spore density to investigate possible heterogeneity in the spore batches using self-establishing Percoll gradients (38). The spores were of uniform density, and dormant spores formed a single band at 1.13 g/ml, comparable to what was previously reported (38). Germinated spores settled between 1.062 and 1.075 g/ml and cell debris settled typically at 1.049 g/ml. The obtained *B. cereus* spores were stable over time and germinated readily upon addition of alanine and inosine. This method of spore production is excellent for production of large, pure, and homogeneous spore batches that are stable over time and suitable for detailed studies.

Gene expression. Sporulation in *B. subtilis* is orchestrated through the compartmentalized action of four sigma factors, σ^F, σ^E, σ^G, and σ^K (30). Sigma factor σ^A is the primary sigma factor in *B. subtilis*, regulating macromolecular synthesis and playing a key role in the housekeeping functions of the cell. The alternative sigma factor σ^B plays a role in the general stress response including entry into stationary phase (16). The *B. cereus* genome is predicted to contain a single homolog to *B. subtilis sigA*, *sigB*, *sigF*, and *sigG* (Table 1). We used quantitative PCR to measure *sigF*, *sigG*, *sigA*, and *sigB* expression during the various growth phases in *B. cereus*. With quantitative PCR the amount of product after each amplification cycle is measured. The obtained data were normalized with a dilution series of genomic DNA. In this way, efficiencies of the specific primer annealing and amplification reactions are taken

TABLE 1. Sigma factor genes from *B. subtilis* and *B. cereus* and their similarities based on amino acid sequence, with ERGO database open reading frame numbers

Sigma factor	ORF[a] no.		% Similarity	% Identity
	B. subtilis	*B. cereus*		
σ^A	2514	856	93	88.5
σ^B	473	5124	73	52.5
σ^F	2341	2311	91	79.8
σ^G	1534	532	94	89.2

[a] ORF, open reading frame.

into account, which allows quantitative comparison of the *sigA*, *sigB*, *sigF*, and *sigG* transcripts.

During phase 1 and the first half of phase 2 the cell density was too low for efficient harvesting. Halfway through phase 2, when the absorbance reached 0.2, the first cell samples were taken and transcripts of *sigA*, *sigB*, and *sigF* were detected (Fig. 1C). Phase 2 was characterized by a high expression of *sigA*, low expression of *sigB* and *sigF*, and the absence of *sigG* transcripts. The *sigA* transcript was most abundant, while the *sigB* and *sigF* expression was 100- and 20-fold lower, respectively. The high-level expression of *sigA*, which continues through phases 3 and 4 (see below), confirms its anticipated pivotal role in all cellular processes. The detection of small amounts of *sigB* and *sigF* transcript during exponential growth may be explained by either some heterogeneity or low expression by the majority of the cells. However, at the end of phase 2, upon entry into early stationary phase (phase 3), transcription of *sigB* and *sigF* increased markedly (see below), to 8- and 30-fold over initial values, respectively, while *sigA* expression remained essentially constant. This indicates that the vast majority of the cells enter stationary phase at the same time, confirming synchronicity of the culture.

Phase 3, the early stationary phase, was characterized by peaks of *sigB* and *sigF* expression, while *sigA* transcription remained high and the first *sigG* transcripts were detected at a low abundance. We detected the *sigB* expression early in phase 3, at the time of glucose exhaustion; subsequently lactate was metabolized (Fig. 1B). In *B. subtilis*, σ^B is expressed upon entry into stationary phase (16) and when the cell encounters stress, directly affecting cell and carbon metabolism (32). Our findings fit this idea, although of the three lactate dehydrogenase genes in the *B. cereus* genome none contains a σ^B consensus sequence, and none are upregulated when σ^B is activated (W. V. Schaik, personal communication). This suggests that, in *B. cereus*, if σ^B influences carbon metabolism, it does so indirectly. *sigF* transcription was upregulated parallel to *sigB* transcription but to a higher level (30-fold, Fig. 1C) and showing a broader peak that lasted through phases 3 and 4. This is before sporulation started, as with *B. subtilis* (8). Halfway through phase 3, *sigG* transcripts could be detected for the first time (Fig. 1C). Initial *sigG* transcription intensity was, like that of *sigB* and *sigF* transcription, much lower than that of *sigA* and remained low during phase 3. Sigma factor σ^G plays a role in the final stages of sporulation. It is expressed in the forespore and regulates synthesis of SASP and Ger proteins (16).

Phase 4, the late stationary and early sporulation phase, was characterized by a significant increase (140-fold) of *sigG* expression. In *B. subtilis* σ^G is, just like σ^F, held inactive until the appropriate moment, while *sigG* transcription is, in addition to stimulation by σ^F, stimulated by active σ^G (30). Indeed, upstream of the *B. cereus sigG* we found a clear σ^G consensus sequence (data not shown) that fits with those previously described (3). This, in combination with the sharp peak of *sigG* expression, shows that in *B. cereus* autostimulation of *sigG* probably takes place. Interestingly, *sigF* transcription remains high in phase 4 after its peak in phase 3. This suggests that, while σ^G is already active, *sigF* is still transcribed or, more likely, the *sigF* transcript is quite stable over time.

In phase 5 the spores became phase bright and microscopically clearly visible (Fig. 1B and 2C). The bulk of the spores acquired a phase-bright appearance soon after the peak in *sigG* expression. This indicates forespore core dehydration in response to σ^G activity, as in *B. subtilis* (8). The oxygen consumption (data not shown) fell to nearly zero, confirming that all metabolic activity stopped, while as anticipated the amount of *sigA*, *sigB*, *sigF*, and *sigG* transcripts markedly decreased in this phase.

In conclusion, we have described an easy and efficient way of producing synchronized and homogeneous *B. cereus* spore batches. The chemically defined medium in combination with the fermentor system allows precise monitoring and manipulation of key growth and sporulation parameters and results in synchronous growth and sporulation, which facilitates gene expression studies. The kinetics of expression of *sigA*, *sigB*, *sigF*, and *sigG* follow the model developed for *B. subtilis*, underscoring the conservation of sporulation mechanisms among bacilli. Future studies will focus on gene expression differences during growth and sporulation with different sets of parameters, for example, pH and carbon source.

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