Effect of the *Bacillus atrophaeus* subsp. *globigii* Spo0F H101R Mutation on Strain Fitness

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Sporulation is a critical developmental process in *Bacillus* spp. that, once initiated, removes the possibility of further growth until germination. Therefore, the threshold conditions triggering sporulation are likely to be subject to evolutionary constraint. Our previous studies revealed two spontaneous hypersporulating mutants of *Bacillus atrophaeus* subsp. *globigii*, both containing point mutations in the *spo0F* gene. One of these strains (Detrick-2; contains the *spo0F101* allele with a C:T [His101Arg] substitution) had been deliberately selected in the early 1940s as an anthrax surrogate. To determine whether the experimental conditions used during the selection of the “military” strains could have supported the emergence of hypersporulating variants, the relative fitness of strain Detrick-2 was measured in several experimental settings modeled on experimental conditions employed during its development in the 1940s as a simulant. The congenic strain Detrick-1 contained a wild-type *spo0F* gene and sporulated like the wild-type strain. The relative fitness of Detrick-1 and Detrick-2 was evaluated in competition experiments using quantitative single nucleotide polymorphism (SNP)-specific real-time PCR assays directed at the C:T substitution. The ancestral strain Detrick-1 had a fitness advantage under all conditions tested except when competing cultures were subjected to frequent heat shocks. The hypersporulating strain gained the maximum fitness advantage when cultures were grown at low oxygen tension and when heat shock was applied soon after the formation of the first heat-resistant spores. This is interpreted as gain of fitness by the hypersporulating strain in fast-changing fluctuating environments as a result of the increased rate of switching to the sporulating phenotype.

Sporulation in Gram-positive *Bacillus* species is a critical developmental process that results in the formation from growing vegetative cells of dormant, extraordinarily hardy, persistent endospores (42). The formation of spores is a complex biochemical process (13, 51) linked to starvation that is irreversible once initiated (53). While entry into sporulation occurs with some heterogeneity within otherwise uniform bacterial cultures (10), entry into sporulation across a bacterial population must therefore be optimized to minimize the likelihood that conditions would improve sufficiently to permit additional growth. Failure to time sporulation appropriately would result in missed opportunities for a population of bacteria when nutrient deprivation is transient. In most sporulating bacteria studied to date, sporulation is regulated by a network of sensor kinases that transmit signals via a phosphorelay cascade (21) to Spo0A (15), a transcription factor that is the master regulator of the sporulation regulon. In *Bacillus subtilis*, the best-studied of sporulating organisms, the Spo0F protein integrates the signals from sensor kinases by accepting a phosphoryl group and relays it to Spo0B (54) and then to Spo0A (23), which directly regulates the expression of as many as 120 downstream genes (39).

Recently, we performed whole-genome molecular typing of several *Bacillus atrophaeus* subsp. *globigii* strains, including those used by the U.S. military as simulants for biological warfare and bioterrorism events (16). The “military” strains were selected in the 1940s (18) for their high spore yield (hypersporulating strains); however, the exact protocols of the original experiments were lost. The whole-genome molecular typing (16) of several extant *B. atrophaeus* isolates from different laboratories that had traceable provenance from Camp Detrick showed that all hypersporulating military strains in use today were descendants from strain Detrick-2, which differed by a single mutation at the H101R

locus of Spo0F from the Detrick-1 variant. This variant was present in the same archival population that could be distinguished from Detrick-2 by its distinctive colony morphology. In the same study, another mutation of Spo0F, at the A98P locus, was found in the group 1013 strains of *B. atrophaeus* subsp. *globigii*, which appeared to have evolved from the ancestral 1942 strain independently of the military lineage.

Mutation of H101 of Spo0F to alanine caused a hypersporulating phenotype in *B. subtilis* (24) by decoupling sporulation from two of the five sensor kinases, presumably by altering the amino residues in the β 4- α 4 recognition loop (38). The Spo0F protein of *B. atrophaeus* subsp. *globigii* is identical to the same protein of *B. subtilis* except for two amino acids. The other components of the phosphorelay share similar degrees of similarity, suggesting that the overall function of the sporulation cascade is highly conserved in *B. atrophaeus* subsp. *globigii*. The independent emergence of hypersporulating variants in two separate lineages of *B. atrophaeus* warrants attention because, despite many reports of *spo* mutations in evolving populations of *B. subtilis*, *Bacillus anthracis*, *Bacillus thuringiensis*, and other spore-forming bacilli, very few reports of spontaneous mutants that sporulate early have emerged (16, 19). The most likely explanation for this observation is that conditions used in other attempts were not optimized to favor hypersporu-

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lating mutants (36). Therefore, we reasoned that the hypersporulating variants of *B. atrophaeus* could serve as an experimental model system to test different environments for their capacity to support the emergence of hypersporulating variants.

For the military strains to have been deliberately selected from cultures used in the 1940s and 1950s, hypersporulating variants should have been present at sufficient frequencies to allow the discovery by visual inspection of distinct colony morphotypes on agar plates. Hayward et al. (19) estimated the presence of the hypersporulating “F” colonies in their plate cultures to be 1 in 1,000. Such a common occurrence of hypersporulating variants in the cultures suggests that the hypersporulating variants were fixed at low frequencies. It is not known under what conditions the military lineage of *B. atrophaeus* was selected; therefore, we decided to reexamine the experimental conditions present in the experiments done in the 1940s and 1950s (19, 47) and determine whether any set of conditions used favors the emergence of the hypersporulating strain Detrick-2 when placed in direct competition with its congenic progenitor strain Detrick-1. Using allele-specific quantitative PCR assays to determine relative strain ratios, we conducted competition experiments between unmarked Detrick-2 and Detrick-1 strains. Under all *in vitro* growth conditions tested, the hypersporulating variant was less fit than the parent strain, save when timed heat shocks were applied to the evolving cultures. We propose a model for analysis of competition experiments using sporulating bacteria that allows the study of the effect of hypersporulation on fitness of early-sporulating variants in rapidly fluctuating environments.

MATERIALS AND METHODS

Bacterial strains and experimental conditions. *B. atrophaeus* subsp. *globigii* strains Detrick-1 and Detrick-2 were used in the experiments. The origin of the strains was described elsewhere (16). Cells were cultured in corn steep liquor (CSL) (Sigma-Aldrich, St. Louis, MO). The stock CSL contained approximately 50% solids and 50% liquid with dissolved water-soluble components (32). The water-soluble components were used for the preparation of culture media as described elsewhere (19, 47). Briefly, the stock CSL was centrifuged at $14,000 \times g$ for 15 min. The supernatant was collected, and its pH was adjusted to pH 7.0 with sodium hydroxide (NaOH). Finally, the supernatant was cleared by performing one additional centrifugation at $14,000 \times g$ for 20 min. The cleared supernatant was used for the preparation of 1.5% (wt/vol) CSL growth medium. Planktonic cultures were shaken on a rotary shaker at 250 rpm and 33°C (MaxQ 4000; Thermo Scientific, Asheville, NC). The cultures were propagated by serial transfer every 24 h with or without the application of a heat shock regimen. Heat-shocked inocula were obtained by heating 0.5 ml of overnight culture on a heat block at 65°C for 30 min (47). The temperature of the inocula during the heat shock was measured with a thermocouple (Fisher Scientific). Sequential cultures were started by diluting the inocula with fresh medium at a 1:200 (vol/vol) ratio. This corresponded to an average of 7.6 generations per 24 h. Lawn cultures were grown on 1.5 wt% (weight percent) agar plates made with 2.5 wt% CSL (19). The cultures were started using 1-ml planktonic cultures grown to an optical density (OD) at 600 nm of 1.0. Two groups of lawn cultures were grown. The lawn cultures from the first group were restarted every 48 h using the following protocol: 2 ml of phosphate-buffered saline (PBS) was added on top of the old lawn culture, and the cells were resuspended in the added buffer. The 2-ml cell suspension was transferred to a 15-ml culture tube and shaken for 15 min on a rotary shaker (250 rpm and 33°C). Then, 1 ml of the cell suspension was used to inoculate one fresh plate and the remaining 1 ml was used for DNA extraction. The lawn cultures from the second group were serially restarted after a 7-day incubation using the same protocol as was used for the 48-h lawn cultures. Two types of inocula

were used to restart the 7-day lawn cultures. The first type of inoculum was obtained using the protocol used for restarting the 48-h lawn cultures. The second type of inoculum was obtained by incubating the inoculum for 12 h with 50% ethanol to kill any vegetative cells (26). The inocula treated with 50% ethanol were washed once with PBS before their use for inoculation of fresh plates.

Each experimental series was started from a single colony. Three-day-old or older colonies were used. The cells from a chosen colony were heat shocked and grown as a planktonic culture to an OD between 1.0 and 2.0 in 1.5 wt% CSL. Then, 0.5-ml samples were taken from the single-strain stock cultures and used to determine the percentage of live cells using Invitrogen's LiveDead BacLight assay (Invitrogen, Carlsbad, CA) and flow cytometry (BD FACSCanto II; BD Biosciences, San Jose, CA) (see Fig. S1 in the supplemental material). Cultures with a 94% or higher percentage of live cells were used as the stock cultures for preparing the mixed starting cultures for competition experiments. The strain frequency of the starting cultures was adjusted using OD measurements.

DNA isolation. Samples for DNA isolation were collected from competition experiments and washed once with PBS and five times with PBS containing 10% dimethyl sulfoxide (DMSO). The DMSO washes removed possible polymerase inhibitors present in the CSL and significantly increased the quality of the isolated DNA. Genomic DNA was isolated using the PowerSoil kit (MoBio Laboratories, Carlsbad CA) according to the manufacturer's protocol with two modifications. First, 10 μ l of proteinase K (10 mg/ml; Sigma) was added to solution C1. The cells were incubated in solution C1 for 10 min at 65°C prior to beating in a bead beater for 40 s. Second, an additional desalting wash with ethanol was introduced after the wash with solution C5. The quality of the DNA obtained was checked with a NanoDrop ND-1000 spectrophotometer (Thermo Scientific).

Real-time PCR. Single nucleotide polymorphism (SNP)-specific forward primers for the Spo0F(H101R) mutation were designed using the sequence information from the whole-genome typing study (16). The forward primers were pSpo0F(101H)-D1-f, matching the Detrick-1 genomic DNA (gDNA) strand (5'-TCAAAAGGCTTAGCGAAcT-3'), and pSpo0F(101R)-D2-f, matching the Detrick-2 gDNA strand (5'-TCAAAAGGCTTAGCGAAcT-3'). One mismatch in the -1 position (lowercase nucleotide) was introduced to improve primer performance. The nucleotide at the 3' position corresponded to the (C:T) SNP of the Spo0F gene (16). One common reverse primer, pSpo0F(H101R)-186-rev (5'-GCCCTGATCTTGTCTCCTC-3'), was used to produce a 186-bp amplicon. Primers specific for *B. atrophaeus* subsp. *globigii* and producing a 228-bp amplicon for the putative RNA helicase (16) were designed and used as internal-control primers. They were pBG21115-f (5'-AACCGTCGTAAA CCAAGTCG-3') and pBG21115-r (5'-TCCAAAGGTTCTGTCCCATC-3'). Three primer sets were assembled using the above-described primers, set pD1 comprised of pSpo0F(101H)-D1-f and pSpo0F(H101R)-186-rev, set pD2 comprised of pSpo0F(101R)-D2-f and pSpo0F(H101R)-186-rev, and set p21115 comprised of pBG21115-f and pBG21115-r.

The PCR reaction mixture volume was 10 μ l, including 500 μ M each primer, a template, and 5 μ l of SsoFast-EvaGreen supermix (Bio-Rad, Hercules CA). PCR amplification and fluorescence detection were carried out on either a LightCycler (Roche) or a CFX-Connect (Bio-Rad), using white PCR plates for the LightCycler. The thermal cycling for the LightCycler included a 5-min initial denaturation at 94°C, annealing/extension for 40 s at 54°C, and fluorescence acquisition at the end of each extension for 45 cycles. The thermal cycling for the CFX-Connect included 5 min of initial denaturation at 94°C, step down to 60°C for 3 s, annealing/extension for 40 s at 54°C, and fluorescence acquisition at the end of each extension for 45 cycles. Melting curve measurements were performed after the completion of cycling for each real-time PCR. The measured cycle threshold (C_T) values using either the LightCycler or the CFX-Connect systems were comparable.

Competition experiments. Two types of growth conditions were considered in competition experiments using broth cultures, sporulation

promoting and sporulation suppressing. The sporulation-promoting conditions were high oxygen tension conditions designed after those used by Roth et al. (47), with expected above-90% sporulation after 24 h of incubation in CSL (47) and an oxygen absorption rate equal to 1.3 mM/min (2). We used 250-ml Erlenmeyer flasks and 6-ml broth cultures to mimic the sporulation-promoting conditions from Roth et al. (47). The oxygen absorption rate for the sporulation-promoting conditions was similar to or higher than the oxygen absorption rate in the experiments of Hayward et al. (19). The sporulation-suppressing conditions were low oxygen tension conditions where the spore yield after 24 h of incubation was expected to be below 1% (47). We used 50-ml Erlenmeyer flasks and 20-ml culture volumes with an expected rate of oxygen absorption equal to 0.22 mM/min (2). The oxygen absorption rate for the sporulation-suppressing conditions was lower than the oxygen absorption rate in the experiments of Hayward et al. (19).

Sporulation-suppressing conditions combined with heat shock timed soon after the formation of the first heat-resistant spores were used for achieving a high fitness advantage for the hypersporulating variant. In this protocol, single-strain stock cultures were started from single colonies as described above. The stock cultures were serially propagated as exponentially growing cultures three times. Serial dilutions were used for the re-starting of the cultures to ensure that we used the minimum number of cells in the inocula. The ratio of Detrick-1 and Detrick-2 strains in the starting cultures was 1:1. After 20 h of incubation, a 1-ml sample was taken from the cultures and heat shocked at 85°C for 30 min. The heat-shocked samples were plated on 2.5% CSL agar plates and incubated at 33°C for 16 to 20 h. Fifty single colonies were randomly selected from the plates, and single-colony cultures were started. gDNA isolated from the single-colony cultures was used to determine the strain frequencies after the timed heat shock. The colonies from the plates, which were not selected for growing single-colony cultures, were incubated for 12 days at 33°C, when they formed dense lawn cultures. The cells and spores from the lawn cultures were scraped, washed, and used for gDNA isolation and determination of strain frequency.

Characterization of sporulation using dark-field microscopy. An Olympus BX51 microscope (Olympus, Japan) equipped with a 100× oil immersion dark-field objective and oil immersion dark-field condenser was used for spore visualization. A CoolSnap HQ2 camera (Photometrics, Vancouver, Canada) controlled by NIS-Elements (Nikon, Japan) was attached to the microscope and used for image acquisition and image analysis. Samples for dark-field microscopy were prepared as follows. Clean microscope slides were coated with polylysine (Sigma) and allowed to dry in air. A drop of cell culture suspension was added to each slide. The slides were covered with coverslips and mounted on the microscope stage for observation.

Determination of strain frequency from PCR data. The strain frequency P_x was calculated from the PCR data using

$$P_x(t) = \frac{(1 + E)^{C_{T,y(t)}}}{(1 + E)^{C_{T,x(t)}} + (1 + E)^{C_{T,y(t)}}} \quad (1)$$

where $P_x(t)$ was the frequency of strain x at time t , E was the efficiency of the PCR, and $C_{T,x(t)}$ and $C_{T,y(t)}$ were the C_T values for strains x and y , respectively.

Alternatively, the logarithm of the frequency ratio plotted against the generation time was used to calculate the rate of change in frequency. This was similar to the calculation of the selection coefficient (9) or selection rate constant (30). However, because the cultures grown here were density dependent and were grown in fluctuating environments, real time was used instead of generation time. The logarithm of the frequency ratio was calculated from

$$\ln \left[\frac{P_x(t)}{P_y(t)} \right] = \ln(1 + E) [C_{T,y(t)} - C_{T,x(t)}] \quad (2)$$

By convention, Detrick-1 was considered to be the x strain and Detrick-2 was considered to be the y strain.

RESULTS

Experimental design. In planning the conditions for the competition experiments, we sought to mimic as closely as possible the experimental conditions used during the development of *B. atrophaeus* subsp. *globigii* as an anthrax surrogate. The selection of an appropriate strain was guided at the time by the following criteria: rapid growth, high spore yield, and experimental reproducibility (19). To satisfy these criteria, *B. atrophaeus* subsp. *globigii* cultures were grown in CSL with shaking and combined with colony selection (19). Later publications from that period clarify the importance of culture aeration for obtaining high spore yields after 24 h of incubation and provide estimates for the rates of oxygen absorption used in those experiments (2, 47). Heat shocks of inocula were used as a purifying selective pressure for the cells that failed to sporulate after 24 h of incubation (19, 47). Based on this information we used the following experimental conditions: (i) growth of cultures in CSL; (ii) modulation of spore yield after 24 h of incubation by using different rates of oxygen absorption; (iii) growth of planktonic cultures or lawn cultures (lawn culture were grown to mimic storage conditions as slants); and (iv) heat shock of inocula after 24 h of incubation.

SNP-specific qPCR assay for competition experiments. The ability to conduct competition experiments required a discriminatory assay that could determine the relative ratios of the strains over a broad dynamic range. Antibiotic-resistant strains have been used in directed evolution and competition experiments of *B. subtilis* (7, 41). To avoid subtle yet potentially confounding perturbations to strain fitness associated with the introduction of an antibiotic resistance cassette, we reasoned that the use of antibiotic-resistant strains would complicate the interpretation of the experimental results. Therefore, we elected to use the (T:C) single nucleotide polymorphism of the *spo0F101* allele that is characteristic of Detrick-2 as a genomic marker to differentiate between the two competing strains. The use of PCR for strain quantitation has advantages over the use of antibiotic resistance cassettes because using kits allowing gDNA extraction from either vegetative cells or spores (11) allows us to account for cells in different phases of their life cycle. Quantitative detection of SNPs in mixed samples is performed by using either endpoint detection methods (22, 37, 49) or real-time quantitative PCR (qPCR) (8, 12, 28, 40, 52, 55). Here, we developed an SNP-specific qPCR assay for quantitation of competition data based on the relative quantification method (33).

Validation of the use of the relative PCR quantification method for analysis of competition experiments. Two PCR methods are commonly applied for analysis of real-time PCR data, the absolute quantification method using standard curves (48) and the relative quantification method using internal controls (50). The relative quantification method has the advantage that it is suitable for the analysis of large volumes of data. Here, the relative quantification method was tailored to the analysis of data from competition experiments. The application of the relative quantification method requires careful assay design in which the efficiencies of all PCRs are close to 100% (33). The primer sets listed in Materials and Methods were designed to fulfill this requirement (see Fig. S2 in the supplemental material).

To evaluate the efficacy of our PCR assays for determining the relative ratios of alleles within a population, we validated the proposed approach using defined mixtures of gDNA from each strain.

FIG 1 PCR-based method to quantify relative abundance of *spoOF* alleles in a mixed population of Detrick-1 and Detrick-2. (A) Real-time PCR traces for the internal control primer set p21115 and the two SNP-specific primer sets pD1 and pD2 for Detrick-1 template (tD1) and Detrick-2 template (tD2). a.u., arbitrary units. (B) Agarose gel showing the products of four PCRs, two for each template. The 50-bp ladder is shown at the left. Two primer sets were used in each reaction mixture, the internal control primer set and one of the SNP-specific primer sets. (C) Measured C_T values for mixed tD1/tD2 templates obtained from single-strain cultures grown in CSL using indicated primer sets. The efficiency of the PCR for each primer set was calculated from the slope of the linear regression approximation of the experimental data. The experimental data shown in red represent the measured C_T values for DNA templates isolated from mixed cell samples. The total amount of template per PCR was 20 ng and was adjusted using the p21115 primer set. (D) Analysis of the hypothetical competition experiments for emergence of Detrick-1 (left) and Detrick-2 (right) strains. The hypothetical experimental selection coefficient was calculated from the slope of $\ln[P_x(t)/P_y(t)]$ versus the hypothetical generation time. theor. fitness param., theoretical fitness parameter.

Figure 1 illustrates key steps of the validation of the relative quantification method. The time courses of three real-time PCRs using the three primer sets introduced in Materials and Methods and templates from either strain Detrick-1 (tD1) or Detrick-2 (tD2) (pure templates) are shown in **Fig. 1A**. The internal-control primer set p21115 was used to determine the total template concentration. The size and GC content of the amplicon generated by the control primer set were chosen such that the corresponding C_T value was close to the C_T value for the matching SNP-specific primer set. This simplified the data analysis significantly because it allowed us to use the two SNP-specific primer sets as internal controls for each other. The difference between the C_T values of the two SNP-specific primer sets when used with pure templates provided an estimate of the assay's selectivity. The selectivity was 1:1,000 for the tD1 template and 1:4,000 for the tD2 template (**Fig. 1A**) (also see the supplemental material). The products from four

PCRs are shown in **Fig. 1B**. Except for the amplicons corresponding to the control primer set and the matching SNP primer sets used in the PCRs, no other products were present. The results in **Fig. 1A** and **B** showed that the primer sets designed were selective and suitable for use with real-time PCR.

We discovered during the development of the assay that CSL contained uncharacterized PCR inhibitors. Therefore, we initially developed the assays using pure templates (see the supplemental material for more detail). Pure templates were obtained from cells grown in LB broth where the presence of polymerase inhibitors was not detected. The effective dynamic range of the assay was determined by varying the ratios of template concentration $[tD1]/[tD2]$ over 4 orders of magnitude (**Fig. 1C**) at a constant total quantity of gDNA per 20-ng reaction mixture. The same figure also shows results from qPCR experiments using gDNA extracted from mixed cell cultures. The dependence of the C_T values on the

ratio of template concentration was similar for both templates obtained from mixing single-strain gDNA isolates or gDNA extracted from mixed cell cultures. This justified the use of the efficiencies measured for templates obtained from mixing single-strain gDNA in the analysis of competition experiments.

The application of the relative quantification PCR method for analysis of competition experiments is illustrated using a hypothetical competition experiment where one of the species emerges in a mixed culture (Fig. 1D). The strain with the smaller initial frequency was considered the emerging strain. When Detrick-2 was the emerging strain, the graph was labeled “Detrick-1 elimination” because, by convention, Detrick-2 was arbitrarily chosen as the y strain in equation 2. One unit of hypothetical generation time was defined as the time necessary for the doubling of the emerging strain. At generation time zero, the frequency of the emerging strain was taken as 0.001. Using these conditions, the theoretical selection coefficient was equal to 0.693. The data from mixed cell samples shown in Fig. 1C were used to calculate the hypothetical experimental selection coefficient. The slope of the dependence of $\ln[P_x(t)/P_y(t)]$ on generation time calculated from the data for the hypothetical emergence of Detrick-1 was 0.619, and the one for hypothetical elimination of Detrick-1 was -0.647 (Fig. 1D). These values were close to the theoretical selection coefficient, which demonstrated the utility of the proposed approach for analyzing competition experiments.

Competitive fitness in the absence of selective pressure for sporulation. Following our experimental design, the cultures were incubated for 24 h. Under the conditions used here, the exponential phase was completed after the first 9 to 10 h of incubation. During the remaining time before the completion of the 24-h incubation cycle, the cultures were in a stationary phase. Therefore, the cultures were subjected to a biphasic fluctuating environment characterized by oscillations between periods of rich and depleted nutrient conditions. To determine which strain had an overall fitness advantage in this fluctuating environment, samples were taken for analysis at the start of the experiment, at the end of the first exponential phase, after the first 24 h of incubation, and after every subsequent 24 h of incubation. The measured logarithms of the frequency ratios for sporulation-promoting and sporulation-suppressing conditions plotted versus time for four serially restarted cultures are shown in Fig. 2. Under both conditions, the hyposporulating strain Detrick-1 had a fitness advantage. The rates of change of frequency estimated from the slopes of the curves in Fig. 2 were $(0.012 \pm 0.002) \text{ h}^{-1}$ for sporulation-promoting conditions and $(0.038 \pm 0.007) \text{ h}^{-1}$ for sporulation-suppressing conditions. After four serial restarts of the cultures, these rates corresponded to reductions of the frequency of Detrick-2 from 0.5 (in the starting cultures) to 0.25 under sporulation-promoting conditions and 0.03 under sporulation-suppressing conditions. This result has implications for the possibility of isolating hypersporulating mutants on the basis of colony morphology: assuming that the ratio of hypersporulating colonies to wild-type colonies was 0.001 in the Camp Detrick cultures, 1 week of serial transfer would have resulted in the depletion of the hypersporulating variant to a frequency of ~ 0.00001 . This is below the limit of detection using colony counts and would have been interpreted as effective purging of the hypersporulating variants from the cultures. We believe that the reported inconsistencies of results derived from cultures grown under variable oxygen tension (19) were in part due to differences in strains' fitness.

FIG 2 Competition experiments for Detrick-1 and Detrick-2 in the absence of selective pressure for spore formation. (A) $\ln[P_x(t)/P_y(t)]$ versus time of incubation under sporulation-promoting conditions. Strain Detrick-1 was chosen to be the x strain and Detrick-2 the y strain from equation 2. The average rate of change of frequency (see the text) calculated from three experiments was $(0.012 \pm 0.002) \text{ h}^{-1}$. (B) $\ln[P_x(t)/P_y(t)]$ versus time of incubation under sporulation-suppressing conditions. The average rate of change of frequency calculated from four experiments was $(0.038 \pm 0.007) \text{ h}^{-1}$.

Competitive fitness in the presence of selective pressure for sporulation. Roth et al. (47) used an experimental protocol in which fresh cultures were started either from spores or from sporulated old cultures. In this protocol, heat shock was used to kill any vegetative cells and induce spore germination (47). We reasoned that the heat shocks may have acted as selective pressure for spore formation, and we therefore introduced recurrent heat shocks into the growth regimen. The changes of strain frequency in serially propagated mixed Detrick-1 and Detrick-2 cultures with application of heat shocks at the time of culture restart are shown in Fig. 3. Under both sporulation-promoting and sporulation-suppressing conditions, the linear regression trend lines had negative slopes, which corresponded to elimination of strain Detrick-1 (see strain convention for equation 2). The rate of change of frequency for sporulation-promoting conditions was $(-0.0417 \pm 0.0021) \text{ h}^{-1}$ (Fig. 3A) and for sporulation-suppressing conditions was $(-0.081 \pm 0.015) \text{ h}^{-1}$. The data in Fig. 3A and B show that when heat shock was applied at the time of reinoculation, the hypersporulating strain Detrick-2 acquired a distinct fitness advantage. Because the hypersporulating strain did not exhibit a fitness advantage in either the exponential phase or the stationary phase (Fig. 2B), we conclude that the acquired fitness advantage was a result of readjustment of strain frequencies after the heat shock.

The following model was developed to evaluate the effect of heat shock on frequency readjustment during serially propagated cultures using heat-shocked inocula. The model allowed us to calculate the ratio of the percentage of heat-resistant/germinating spores of the two competing strains present in the culture just before the application of heat shock. At the beginning of the ex-

FIG 3 Inclusion of heat shocks in the growth regimen enriches for hypersporulating mutations. Serial transfers of 24-h cultures were performed using heat shock for purging the inocula from the vegetative cell population. Strain Detrick-1 was chosen to be the x strain and Detrick-2 the y strain from equation 2. (A) (Left) $\ln[P_x(t)/P_y(t)]$ versus time of incubation under sporulation-promoting conditions. The average rate of change of frequency from three independent experiments was $(-0.0417 \pm 0.0021) \text{ h}^{-1}$. (Right) Model for acquisition of fitness advantage by Detrick-2. The calculated ratio of heat-resistant/germinating Detrick-2/Detrick-1 spores at state VS_j was 3.6. (B) (Left) $\ln[P_x(t)/P_y(t)]$ versus time of incubation under sporulation-suppressing conditions. The average rate of change of frequency from three independent experiments was $(-0.081 \pm 0.015) \text{ h}^{-1}$. (Right) Model for acquisition of fitness advantage by Detrick-2. The calculated ratio of heat-resistant/germinating Detrick-2/Detrick-1 spores at state VS_j was 17.4. (C) (Left) Dark-field microscopy images from single-strain cultures after 24 h of incubation under sporulation-promoting conditions. (Right) Percentage of sporulating cells incubated for 24 h at different rates of oxygen absorption measured from dark-field microscopy images (averages from 14 images). Rate of oxygen absorption equal to 0.2 mM/min corresponded to sporulation-suppressing conditions, and rate of 1.3 mM/min corresponded to sporulation-promoting conditions. Error bars show standard deviations.

periment, the culture was at the initial state, labeled "I" (Fig. 3), where the frequencies of the two competing strains were equal to 0.5. During the first 24 h of incubation, the culture was growing as a competing culture in the absence of selective pressure for spore formation until it reached state VS_1 (Fig. 3), which contained both vegetative cells and spores. These conditions favored strain Detrick-1 with a rate of frequency change equal to $(0.012 \pm 0.002) \text{ h}^{-1}$ for sporulation-promoting conditions and $(0.038 \pm 0.007) \text{ h}^{-1}$ for sporulation-suppressing conditions (Fig. 2). State VS_1 was also the state when samples were collected for gDNA isolation. At state VS_1 , vegetative cells were killed by applying a heat shock, which corresponded to transition to state S_1 (Fig. 3). The frequencies of heat-resistant/germinating spores at state S_1 determined

the starting frequency of the culture growing from state S_1 to state VS_2 . The sequence of visiting states VS_j and S_j was repeated until the experiment was terminated. Strain frequencies were measured for samples collected at states VS_j , where both vegetative cells and spores were present. The frequencies of the heat-resistant/germinating spores at states S_j were extrapolated from the measured frequencies at states VS_{j+1} and the measured rates of frequency change from the data in Fig. 2 for the corresponding growth conditions. The measured frequency ratio at state VS_j and the calculated frequency ratio at state S_j were used to calculate the ratio of heat-resistant/germinating spores from each species present at state VS_j at the time of application of heat shock as follows:

$$\frac{\% \text{spores (Detrick-2)}}{\% \text{spores (Detrick-1)}} = \left[\frac{P_x(t)}{P_y(t)} \right]_{VS_j} \cdot \left[\frac{P_y(t)}{P_x(t)} \right]_{S_j} \quad (3)$$

where $[P_x(t)]_{VS_j}$ and $[P_y(t)]_{VS_j}$ were the measured strain frequencies at state VS_j (including both vegetative cells and spores) and $[P_x(t)]_{S_j}$ and $[P_y(t)]_{S_j}$ were the strain frequencies of heat-resistant/germinating spores at state S_j , which restarted the culture after the heat shock.

The calculated ratios of the heat-resistant/germinating spores using the data in Fig. 3 were 3.6 for sporulation-promoting conditions and 17.4 for sporulation-suppressing conditions. For non-interacting genotypes or when only “soft” selection was present (30), the percentage of heat-resistant/germinating spores from each strain in a competing culture was expected to be similar to the percentage of heat-resistant/germinating spores in single-strain cultures grown under similar conditions. We used dark-field microscopy to measure the percentages of sporulating cells in 24-h single-strain Detrick-1 and Detrick-2 cultures (Fig. 3C). The rate of oxygen absorption was varied from 0.2 to 1.3 mM/min. The data in Fig. 3C show that the sporulation of Detrick-2 was much less sensitive to a decrease of oxygen tension than the sporulation of Detrick-1. Sporulation-promoting conditions were characterized by rapid oxygen exchange, while low oxygen exchange rates suppressed sporulation. Under sporulation-promoting conditions, the percentage of sporulating Detrick-1 cells was $12.2\% \pm 2.9\%$ and the percentage of sporulating Detrick-2 cells was $38.2\% \pm 2.7\%$. Detrick-2 therefore sporulated at 3.1 times the frequency of Detrick-1 under sporulation-promoting conditions. Similarly, under sporulation-suppressing conditions, the percentage of sporulating Detrick-1 cells was $3.8\% \pm 1.1\%$ and the percentage of sporulating Detrick-2 cells was $34.6\% \pm 3.7\%$, which represented a 9.1-fold higher percentage of sporulating cells for Detrick-2. These results were consistent with previous studies (16, 19).

The ratio of the percentages of sporulating Detrick-2/Detrack-1 cells calculated from dark-field microscopy images and the ratio of the percentages of heat-resistant/germinating spores calculated from equation 3 were different. The calculated ratio of heat-resistant/germinating spores from equation 3 was larger than the corresponding ratio of sporulating cells calculated from dark-field microscopy images. This suggested that the fraction of heat-resistant spores in the Detrick-2 spore population was larger than the same fraction in the Detrick-1 spore population. This difference was more apparent when sporulation-suppressing conditions were used. We concluded that, in addition to the decrease of the percentage of sporulating Detrick-1 cells under low oxygen tension conditions observed from dark-field microscopy images, the percentage of heat-resistant/germinating spores was further decreased. The percentage of sporulating cells is also dependent on time after completion of exponential growth. We measured the percentages of sporulating Detrick-1 and Detrick-2 cells in single-strain cultures 10 to 14 h after completion of exponential growth (T_0) and found that the percentage of sporulating cells decreased by 35% for Detrick-1 and 25% for Detrick-2 between 10 and 14 h after T_0 (Fig. 4). Taken together, these results suggested that the hypersporulating strain was expected to gain maximum fitness advantage in cultures grown at low oxygen tension when heat shock was applied soon after the beginning of sporulation.

The examination of publically available literature that describes the experiments conducted in the 1940s and 1950s showed that spore enumeration was performed by autolyzing cultures

FIG 4 Quantitation of sporulating cells and rapid enrichment for hypersporulating strains. (A) Percentages of sporulating cells in single-strain cultures versus incubation time as stationary cultures under sporulation-promoting conditions. Error bars show standard deviations. (B) Logarithm of fluorescence intensity versus cycle number for four experiments where heat shock was applied 10 h after completion of exponential growth. The pD1 primer set was used for all PCRs whose results are shown. Dashed lines represent reactions where a 100% matching template (left) or 100% mismatching template (right) was used. Dotted line represents the initial mixed culture containing 1:1 Detrick-1/Detrack-2 cells. The four solid traces represent the experimental data from four cultures started using the initial culture shown with a dotted line. The cultures were heat-shocked 10 h after completion of exponential growth. The heat-shocked inocula were plated and grown on plates for 12 days. The cells from the plates were collected, and gDNA was isolated and characterized by qPCR (see the text and Table 1 for details). Ten nanograms of total gDNA was used in each PCR reaction mixture.

overnight, heat shocking the purified spore preparations, and plating them for colony counting (47). Roth et al. studied the effect of oxygen tension and incubation time on sporulation (47). In those studies, spores were obtained from cultures grown under conditions similar to the sporulation-suppressing conditions considered here. Therefore, we hypothesized that hypersporulating strain frequencies could have increased under these conditions, which would have increased the chances for their emergence. To test this hypothesis, we used a starting culture of 1:1 Detrick-1/Detrack-2, which was serially restarted two times as an exponentially growing culture. After the last restart, the culture was grown until 10 h after the completion of exponential growth, when it was heat shocked and processed as described in Materials and Methods. Forty-nine of the randomly selected 50 single-colony cultures after the heat shock had Detrick-2 signatures. The results from the plate analysis are shown in Fig. 4 and Table 1. The calculated frequencies of Detrick-1 were in the range from 0.003 to 0.016 (Table 1). The results from this experiment confirmed the possibility that variants with hypersporulating phenotypes could have emerged during the early experiments performed in the 1940s and 1950s under poorly controlled aeration conditions (19) or under conditions with varied oxygen tension (47).

TABLE 1 Measured C_T values using different primer sets and calculated frequencies for Detrick-1 and Detrick-2 strains from the experiment providing for a high fitness advantage for the hypersporulating strain (see Materials and Methods)

DNA source	C_T value using primer set:		Frequency of ^a :	
	pD1	pD2	Detrick-1	Detrick-2
Detrick-1 only	17	26.3	0.999	0.001
Detrick-2 only	26	16.4	0.001	0.999
1:1 Detrick-1/Detrick-2	18.2	17.9	0.65	0.45
Expt ^b				
1	24.4	16.6	0.003	0.997
2	24.8	17.2	0.003	0.997
3	22.4	16.5	0.012	0.988
4	22.6	17.1	0.016	0.984

^a The frequencies were calculated using equation 1 and 110% efficiency.
^b Experiments 1 to 4 represent the qPCR analysis of four parallel cultures started from the same initial 1:1 Detrick-1/Detrick-2 culture. The cultures were grown until 10 h after the completion of exponential growth and then were heat-shocked at 85°C for 30 min. Heat-shocked inocula were plated and grown for 12 days, at which time all cells from the plates were collected and used for gDNA isolation and qPCR quantification.

Competitive fitness in lawn cultures. Competition in lawn cultures was carried out to mimic the conditions present during the storage of the archival cultures on slants. Short-term (48 h) and long-term (7 days) serially restarted lawn cultures were used (Fig. 5). The inocula for the 7-day lawn culture were either untreated or treated with 50% ethanol to remove the surviving vegetative cells. The rate of change of strain frequency of the 48-h cultures was on the order of 0.04 h^{-1} , which was similar to the rate of change of strain frequency for the cultures grown under conditions of low oxygen tension without heat shock. The rates of change of frequency of the lawn cultures grown for 7 days were $(0.0026 \pm 0.00009)\text{ h}^{-1}$ for cultures restarted with untreated inocula and $(0.0034 \pm 0.00042)\text{ h}^{-1}$ for cultures restarted with inocula treated with ethanol.

DISCUSSION

Bacteria use diverse strategies for survival in changing environments, including genomic alterations (6, 27), phenotypic switching (3), and mobilization of cooperative responses (5). Sporulation is a classical example of phenotypic switching in fluctuating environments signaled by starvation (53). Mutations in key pathway regulators are rare events, but they represent an important mechanism for increasing phenotypic diversity (29). Recent directed evolution studies combined with whole-genome sequencing have shown that relatively small numbers of mutations in key regulators appearing during the first generations under new selective pressure account for most of the gains in fitness and that subsequent mutations often have diminishing effects on cumulative fitness gains (4, 20, 34, 60). The acquisition of two independent mutations in Spo0F of *B. atrophaeus* subsp. *globigii*, where each mutation gives rise to a hypersporulating variant (16), is one such example of phenotype-defining mutation in a key pathway regulator. The effect of beneficial mutations in key pathway regulators on strain fixation has been studied for the sigma factor σ^S protein encoded by the *rpoS* gene of *Escherichia coli*. *E. coli* switches to a hypermutable state during starvation (43). The high rate of independent mutations of *rpoS* generates strains with a fitness advantage, which is observed as periodic selection sweeps

through experimental cultures (25). Our experiments cannot be compared directly to chemostat experiments because chemostats operate under conditions of constant nutrient input, whereas the conditions in our cultures fluctuate. However, the significant gain of fitness of the hypersporulating strain Detrick-2 leading to a frequency increase from 0.5 to 0.99 after just one timed heat shock in the example shown in Fig. 5 could be considered a culture sweep in a fluctuating environment. To the best of our knowledge, this is the first demonstration of culture sweep by a hypersporulating bacterial strain.

In contrast to the relative paucity of reports of spontaneous mutations conferring a hypersporulating phenotype, many reports have been made of *spo* mutations in evolving populations of *B. subtilis*, *B. anthracis*, *B. thuringiensis*, and other spore-forming bacilli (16, 19, 35, 45, 59). This is most likely because the broadly adopted culturing conditions do not favor the emergence of hypersporulating variants. Another explanation for the failure to isolate such mutants is that, in some cases, sporulation might have already been optimized (36). The isolation on multiple occasions of hypersporulating mutants suggests that this is unlikely to describe *B. atrophaeus*. The experiments performed here can be divided into two groups: (i) experiments performed in two-phase

FIG 5 Competition in lawn cultures incubated for 48 h and restarted with untreated inocula (A), incubated for 7 days and restarted with untreated inocula (B), and incubated for 7 days using inocula treated with 50% ethanol (C).

fluctuating environments, including exponential phase and stationary phase, and (ii) experiments performed in three-phase fluctuating environments, including exponential phase, stationary phase, and a phase of vegetative-cell killing (either via heat shock or treatment with 50% ethanol). The two-phase fluctuating environments represent commonly occurring natural conditions, while the inclusion of high-temperature heat shocks represents a scenario uniquely associated with human intervention that would (under most circumstances) be unlikely to occur in nature. The 24-h broth cultures without heat shock and the 48-h lawn cultures represent simple two-phase fluctuating environments with transient deprivation of nutrients where the switching of the vegetative phenotype to sporulation is incomplete. The 7-day lawn cultures represent conditions of permanent nutrient deprivation where the switching of the vegetative phenotype to sporulation is complete. In all two-phase fluctuating environments, Detrick-1 had a fitness advantage. Detrick-2 gained a fitness advantage in the three-stage fluctuating environment where the switching from vegetative to sporulating phenotype was not complete. The fitness gain was the largest when heat shocks that purged the vegetative cell population were applied soon after the formation of the first heat-resistant spores. The results presented here are examples of gain of fitness as a result of a mutation-controlled rate of switching to sporulating phenotype, which provided a significant fitness advantage in fast-changing fluctuating environments (1).

Our data provide clues regarding the role of growth conditions in the selection of sporulation phenotypes. The entry into sporulation is dependent on a complex cascade of signaling events (21). Mutations deliberately introduced in the members of this cascade have in many cases resulted in the generation of hypersporulating variants (14, 15, 24, 46, 57). In fact, mutation of the analogous H101 position in the Spo0F protein of *B. subtilis* also produces hypersporulating variants (24). Spo0F is a phosphotransferase that shuttles phosphoryl groups from several kinases, including KinA and KinB, to Spo0B and then to the transcription factor Spo0A. Depending on the level of Spo0A phosphorylation, cells enter into either biofilm development, cannibalism (at lower levels of phosphorylation), or sporulation (at high levels of phosphorylation) (15, 21). The ability to produce either hypo- or hypersporulating variants by introducing mutations in the residues surrounding the β 4- α 4 loop (24) suggests that such mutations act as tuning switches. The mechanism of this tuning is unclear. For example, H101 is involved in both binding of Spo0F to kinases (24) and binding to Rap phosphatases (44), which could affect either the transfer of phosphoryl groups from kinases or the regulation of Spo0F dephosphorylation by phosphatases. Alterations to the *spo0F* gene likely decouple the interaction of Spo0F with the sensor kinases or phosphatases, in contrast with the hypersporulating phenotype of a *comK* deletion mutant, which results in de-repression of sporulation-promoting genes, such as *spo0A* (39, 46). Mutants with mutations in the *sdp* and *skf* operons (which are controlled by Spo0A) were shown to sporulate early and also had compromised fitness. The fitness losses in hypersporulating *skf* mutants were shown to be due to active killing (cannibalism) of the *skf* mutants by wild-type cells (17). It is likely that the *skf/sdp* hypersporulating mutants enter sporulation early because of the inability to cannibalize and obtain nutrients rather than as a result of perturbation to the regulatory network governing sporulation. We examined plates containing mixed colonies of Detrick-1 and Detrick-2 and used the test from González-Pastor et al. (17) for

evidence of cannibalistic behavior. We did not observe any cannibalistic behavior (not shown). Overall, our results suggest that any fitness gains deriving from activation of the *skf/sdp* genes (17) by a hyperactive sporulation regulatory cascade would be outweighed by the fitness losses due to the opportunity costs of premature commitment to sporulation by a significant subpopulation of the hypersporulating strain (58) and that it is unlikely that activation of cannibalism under unknown growth conditions would be responsible for the emergence of the Detrick-2 strain at Camp Detrick in the 1940s-to-1950s time period.

Regardless of the underlying mechanism, our data provide insights into the mechanisms of selection between strains with differently tuned entry into sporulation. At the individual cell level, the decision for entry into sporulation is a stochastic process (31, 56) that is partially influenced by the state of the mother cell (58). For continually propagating cultures, premature entry into sporulation by individual cells is disadvantageous because it terminates the cell's ability to continue to divide. This is also true for competing strains where the strain entering into sporulation at the higher rate is at a disadvantage. For cultures grown under conditions of frequent killing of the vegetative cells where new growth is dependent on germinating spores, a higher rate of entry into sporulation could provide a competitive advantage. However, this competitive advantage is limited to cultures where growth is terminated before complete nutrient deprivation. When culture growth is terminated as a result of complete nutrient deprivation, the slowly sporulating strain regains its competitive advantage.

This is the first competition study where Gram-positive bacteria are considered in all stages of their life cycle, including growth, sporulation, and germination, and which identifies early sporulation as a trait that supports the acquisition of a fitness advantage in rapidly changing fluctuating environments. The ability to include bacteria in all stages of their life cycles became possible with the introduction of a novel SNP-specific qPCR assay that uses gDNA extracted from both vegetative cells and spores for strain quantitation. The assay is designed to allow for calculation of strain frequencies with minimum external calibrations, which makes it suitable for high-throughput screening. The assay selectivity is larger than 1:1,000, which makes it suitable for detection of the strains carrying the H101R mutation in environmental samples. A model is developed for calculating the ratio of the percentages of heat-resistant/germinating spores in competition experiments, which provides valuable information for future studies of the development of spore resistance in mixed bacterial cultures.

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