Evolvability of Sporulation and Germination in *Bacillus Subtilis* Batch Culture

Elizabeth Ouanemalay, Kathleen Sagarin, Chloe De Palo, and Fred Cohan

Department of Biology, Wesleyan University, Middletown, CT 06459

Introduction

Endospore-forming bacteria can be found living everywhere from soil, to water, to dust, and to even the human digestive tract.[1,7,3] Endospore formation, or sporulation, is triggered by stressful conditions such as nutrient deprivation, and allows bacterial cells to survive thousands of years in dormancy.[2] However, not all cells in a population will undergo sporulation, even under stressful conditions. This incomplete efficiency appears not to be able to be improved through selection.[4,6] However, the dynamics of sporulation, such as triggers and timing, are genetically based and selectable.[5]

Despite previous work seeing no improvement in sporulation efficiency after two years of selection, this concept is worth revisiting.[4] The original study did not have a control group, only measured sporulation efficiency at one time point, and did not check for improved germination ability , which was likely also under selection in their experiment.[4] Furthermore, the evolution of sporulation and germination dynamics in routine batch culture is unknown.

This study followed *B. subtilis* cell growth and sporulation over 2.3-day periods of batch culture under selection for increased sporulation efficiency, and a non-selected control group . Selection for increased sporulation efficiency was done by transferring only spores between batches, whereas vegetative cells were also transferred in the control group. By measuring growth and sporulation curves we were able to capture evolutionary changes in growth and sporulation dynamics within just a four-week period.

References

1. Driks, A., and Eichenberger, P. 2016. The Bacterial Spore.
2. Higgins, D., and Dworkin, J. 2012. Recent progress in Bacillus subtilis sporulation. FEMS Microbiol Rev *36*, 131–148.
3. Jeżewska-Frąckowiak, J., Seroczyńska, K., Banaszczyk, J., Woźniak, D., Żylicz-Stachula, A., and Skowron, P.M. 2018. The promises and risks of probiotic Bacillus species. Acta Biochim Pol *65*, 1–11.
4. Maughan, H., and Nicholson, W.L. 2004. Stochastic Processes Influence Stationary-Phase Decisions in Bacillus subtilis. Jb *186*, 2212–2214.
5. Mutlu, A., Kaspar, C., Becker, N., and Bischofs, I.B. 2020. A spore quality-quantity tradeoff favors diverse sporulation strategies in Bacillus subtilis. Nature Publishing Group *14*, 2703–2714.
6. Veening, J.-W., Stewart, E.J., Berngruber, T.W., Taddei, F., Kuipers, O.P., and Hamoen, L.W. 2008. Bet-hedging and epigenetic inheritance in bacterial cell development. Proc. Natl. Acad. Sci. U.S.a. *105*, 4393–4398.
7. Zeigler, D.R., and Nicholson, W.L. 2017. Experimental evolution of Bacillus subtilis. Environ Microbiol *19*, 3415–3422.

Results

Results from Selection Experiment

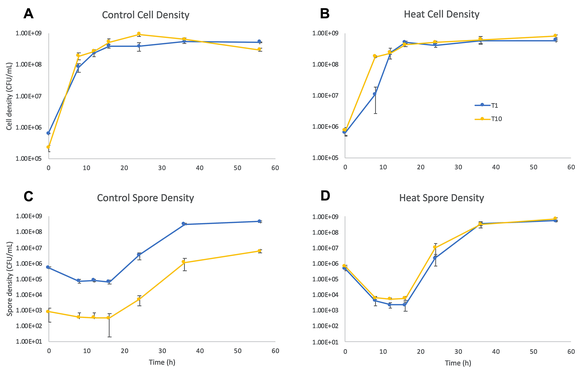

Figure 1: Changes in cell and spore dynamics during the selection experiment. A&C, show changes in spore and cell density in the control group. B&D, show changes in spore and cell density in the heat treatment group. T1: first transfer (before evolution). T10: last transfer (after 4 weeks of evolution). CFU/mL: colony forming unit per milliliter.

Results from Selection Experiment

Sporulation

- During the first transfer, sporulation efficiency (roughly, spore density at 56h) was comparable between the Heat treatment and control groups
- During the tenth transfer, the isolate from the control treatment showed an extreme reduction in sporulation efficiency at 56h, from 90% at T1 to 2% at T10 (fig.1C)

Cell growth

- During the first transfer (T1), cell growth was noticeably delayed in the heat treatment group replicates at 8 hours compared to the control group (fig.1A&B)
- During the tenth transfer (T10), cell growth was broadly comparable between treatments (~0-24h) (fig.1A&B)

Germination

- During the first and last transfers, germination was more efficient in the heat treatment group than the control group (fig.1C&D)
- No significant increase in germination efficiency was measured in the heat treatment over the time points tested (fig.1D)

Results from Preliminary Isolate Testing

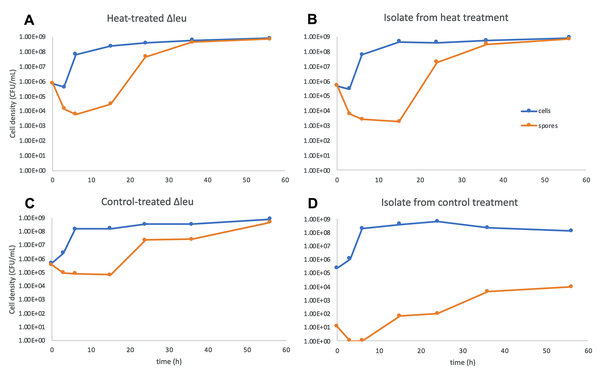

Figure 2: Comparison of ancestral strain, and isolates from evolved communities. A&C, show cell and spore dynamics in the ancestral strain. B shows cell and spore dynamics of an isolate from the heat treatment group. D shows cell and spore dynamics of an isolate from the control treatment group. CFU/mL: colony forming unit per milliliter.

Results from Preliminary Isolate Testing

- The ancestor strain (Δleu) recapitulated the results of T1, with greater germination and delayed growth in the heat treatment compared to the control (fig.2A&C)
- The isolate from a heat treatment replicate shows slightly better germination ability (0-3h) and delayed onset of sporulation (15h) when compared to the ancestor (fig.2A&B)
- The isolate from the control treatment shows an extreme reduction in sporulation efficiency (fig.2D)

Discussion

- No significant increase in sporulation efficiency was seen in the heat treatment, replicating the results of Maughan and Nicholson (2004).
- However, the significant decrease in sporulation efficiency in the control group implies that the heat treatment is acting as stabilizing selection for sporulation ability.
- The delay in growth at the first transfer in the heat treatment is presumably due to germination timing and efficiency (the proportion of spores which germinate).
- However, this delay in growth vanished by the fourth transfer (data not shown).
- It is likely that an increase in germination ability offset the delay in growth, with some supporting preliminary evidence from the isolates.
- This work highlights the importance of a multidimensional approach to research and always including a control group.

Materials and Methods

Strain

- Our ancestral strain was BKK28260 (ΔleuC::kan trpC2) from the *Bacillus* Genetic Stock Center, a derivative of *Bacillus subtilis* 168.

Evolution microcosms

- Eight microcosms were established from a single overnight culture of BKK28260 in Schaeffer's Sporulation Medium (SSM) with kanamycin.
- Microcosms were transferred in serial batch culture every 2.33 days for a total of 11 transfers or ~110 generations.
- Samples of all replicates were frozen in 25% glycerol at T0, T4, T7 and T11 at -80° C.

Tests of growth, sporulation, and germination

- Total cell density and spore cell density were determined at transfer, and specific time points after transfer (experimental microcosms: 8, 12, 16, 24, and 36h; isolates: 3, 6, 15, 24, 36, and 56h).
- Total cell density was obtained by diluting directly from the experimental microcosm and plating onto LB agar plates.
- Spore cell density was obtained by taking a 1mL sample of the experimental microcosm, heating it at 80° C in a water bath for 10 minutes, and using the heated sample for dilution and plating.

Isolates

- Isolates were obtained from samples frozen at the end of the 11th transfer.
- Frozen samples were used to inoculate SSM in an overnight culture, then plated onto LB plates before and after heating for spores.
- After ~24h of incubation, individual colonies were then streaked onto LB agar plates.
- Individual colonies from the isolate plates were then vortexed in 500μL of SSM, to which 500μL of 50% glycerol was added, and then split between two tubes before freezing at -80° C.

Acknowledgements

We would like to thank Fatai Olabemiwo for support in the lab, and Dr. Daniel Zeigler at the BGSC for providing our ancestral strain. We also want to thank the WesMaSS and the John and Rosemarie Dooley Foundation for their generous research funding support.