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Minireview

Experimental evolution of *Bacillus subtilis*

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Summary

The endospore-forming bacteria have persisted on earth perhaps 3Ga, leveraging the flexibility of their distinctive lifestyle to adapt to a remarkably wide range of environments. This process of adaptation can be investigated through the simple but powerful technique of laboratory evolution. Evolved strains can be analyzed by whole genome sequencing and an array of omics technologies. The intensively studied, genetically tractable endospore-former, *Bacillus subtilis*, is an ideal subject for laboratory evolution experiments. Here, we describe the use of the *B. subtilis* model system to study the adaptation of these bacteria to reduced and stringent selection for endospore formation, as well as to novel environmental challenges of low atmospheric pressure, high ultraviolet radiation, and unfavourable growth temperatures. In combination with other approaches, including comparative genomics and environmental field work, laboratory evolution may help elucidate how these bacteria have so successfully adapted to life on earth, and perhaps beyond.

Introduction

Endospore-forming bacteria are among the most globally dispersed of all organisms. They have been isolated everywhere from the upper layers of the atmosphere to the depths of ocean trenches, and from almost every conceivable environment in between (Fajardo-Cavazos *et al*., 2014; Zeigler and Perkins, 2015). Members include not only mesophiles, but acidophiles (Ciuffreda *et al*., 2015), alikaliphiles (Preiss *et al*., 2015), halophiles (Hanelt and Muller, 2013), psychrophiles (Larkin and Stokes, 1966), thermophiles (Zeigler, 2014; Hussein *et al*., 2015) and UV-radiation tolerant organisms (Ordonez *et al*., 2009). Their physiological capabilities suggest they play a significant role in global carbon, nitrogen and sulphur cycles and in the degradation of plant, fungus and insect biomass (Mandic-Mulec and Prosser, 2011). Their ease of isolation and cultivation made endospore-formers among the first bacteria to be studied in pure culture (Ehrenberg, 1835) and a popular subject of microbiological research ever since. Yet their very familiarity may obscure the remarkable evolutionary success story they represent.

Reconstructions of the Tree of Life reveal that endospore formation is extremely ancient, perhaps arising near the phylogenetic root of the bacteria (Tocheva *et al*., 2016). Dating the emergence of taxa in the absence of a fossil record is notoriously difficult. However, one phylogenomics-based method suggests that the last common ancestor of the *Bacilli* and *Clostridia*, two classes encompassing many endospore-forming species, lived 2.87–3.15 Ga (David and Alm, 2011). *Bacilli* and *Clostridia* accomplish sporulation by a similar sequence of developmental events (Dürre, 2014), and their genomes share a set of orthologous genes presumed to be essential for sporulation (Galperin *et al*., 2012; Bate *et al*., 2014), so it seems likely that their ancient ancestor could form endospores (Hutchison *et al*., 2014) – and it may, in fact, have been a spore that survived an early extinction event (Tocheva *et al*., 2016).

Doubtless, the feature most responsible for the 'staying power' of the endospore-formers is the spore itself. The formation and physical properties of endospores have studied intensely due to their importance in medicine, agriculture, food safety and biosafety, as well as their appeal to basic research (Driks and Eichenberger, 2016). In *Bacilli*, sporulation commences when

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nutritional conditions limit growth. Duplicated chromosomes anchor to the cell poles, and an asymmetric septum, laid down near one pole, destines one of the chromosomes to be packaged in the spore. The septum grows around and eventually engulfs the developing spore, or forespore. The mother cell and forespore compartments cooperate to finish the spore's construction. The inner regions become dehydrated and mineralized, and the chromosome becomes complexed with protective proteins. The spore is surrounded by two protective shells, a cell-wall like structure called the cortex, and a multi-layered proteinaceous coat (Tan and Ramamurthi, 2014). In some species the spore is surrounded by an exosporium, a loose-fitting, proteinaceous balloon-like structure through which the spore interacts with its environment (Stewart, 2015). Eventually, the mother cell lyses to release the mature spore. The result is a remarkably tough, heavily protected, metabolically inert cell that can lie dormant until conditions favour growth. The spore's structure confers a high level of resistance to many potentially damaging environmental factors, both biotic and abiotic (Setlow, 2014). Its size and surface properties also make it easily dispersed by wind and water (Nicholson, 2004).

In addition to endospore formation, these bacteria possess a suite of developmental options in response to environmental stresses, and these also should be considered in evaluating the evolutionary success of what one might term the 'Bacillus lifestyle'. Within a stationary phase culture of *Bacillus subtilis*, subpopulations of specialized cells appear under the control of several master regulators of gene expression (Lopez *et al*., 2009). A small fraction of cells enter a unique physiological state– the 'K-state' – in which growth and septum-formation are arrested, DNA repair and recombination systems are activated, and DNA-uptake machinery is assembled (Berka *et al*., 2002). In consequence, K-state cells can potentially acquire DNA from the environment and incorporate it into their genomes. Cells can eventually emerge from the K-state and embark on a program of sporulation (Veening *et al*., 2006). The rest of the stationary phase culture further splits into subpopulations of individual motile cells and end-to-end chains of sessile cells (Mukherjee and Kearns, 2014). A fraction of the non-motile cells transiently secrete massive quantities of antimicrobial compounds (Nonejuie *et al*., 2016) and degradative enzymes, including proteases, nucleases and amylases (Veening *et al*., 2008b). Some sessile chains secrete an extracellular matrix that binds the differentiating cells into biofilms (Claessen *et al*., 2014). A mature *B. subtilis* colony is revealed under magnification to be a remarkable assemblage of cooperating cells, with motile cells near the base, sporulating cells at the top, and matrix-producing chains clustered throughout (Vlamakis *et al*., 2008).

The Bacillus lifestyle, then, refers to the potential for subpopulations within a clonal population to choose from a variety of developmental options, including the formation of cell types that either promote endospore formation or provide alternatives to it. It is easy to imagine how different facets of this lifestyle confer reproductive advantages to the organism. Cells multiply rapidly under ideal conditions, with generation times in the laboratory as short as 30–35 min in *B. subtilis* and approaching 20 min in the related genus *Geobacillus* (D. R. Zeigler, unpublished data). In nature, however, bacteria face nutrient-depleted conditions frequently. The emergence of specialized cell types in response has been interpreted as a bet-hedging strategy that increases the chance that viable cells will survive the crisis to resume proliferation (Veening *et al*., 2008a; Norman *et al*., 2015). The K-state, for example, would be poorly adapted to a nutrient-rich environment. Yet these growth-arrested *B. subtilis* cells in laboratory tests show much greater resistance to antimicrobial compounds, which would likely be produced by neighbours themselves facing starvation, potentially increasing the likelihood that the cell and its genome will survive (Yuksel *et al*., 2016). The transformation competence associated with the K-state could further enhance the cell's survival chances, whether it imports homologous DNA and repairs its genome by recombination or imports heterologous DNA and acquires potentially beneficial genetic traits (Norman *et al*., 2015), or possibly simply uses DNA as a nutrient (Johnston *et al*., 2014). Other specialized cell types may serve an altruistic function, engaging in behaviour detrimental to themselves but beneficial to the population. Subpopulations that secrete massive quantities of degradative enzymes, for example, make a large investment of resources, but as a result may increase the availability of amino acids, simple sugars, and nucleotides for the use of their neighbours as well as themselves (Veening *et al*., 2008b). Such ideas are certainly suggestive as potential explanations for the success of the Bacillus lifestyle, but most have not been rigorously tested either in the laboratory or the field (Grimbergen *et al*., 2015).

These considerations, although briefly stated, raise compelling questions for environmental and evolutionary microbiology. Which features of this lifestyle cooperate to produce reproductive and survival advantages, and in what environments are they advantageous? How has the underlying framework of growth and endospore formation been adapted to such varied environments? How can this adaptability be harnessed for biotechnological purposes? And given the likely transport of bacteria to other solar system bodies (Nicholson, 2009), can the

Bacillus lifestyle allow endospore-formers to adapt to extraterrestrial environments?

Investigating such questions requires a multi-pronged approach, including both field work and comparative genomics. Recently, interest has grown in a third approach, the use of laboratory-based evolution of model bacteria as a means to test hypotheses in evolutionary ecology (Elena and Lenski, 2003). Bacteria are able to reproduce rapidly, allowing thousands of generations to be observed under selection in a short time within a compact laboratory space. Results can be monitored real time. If the experiments are conducted with *B. subtilis*, powerful genetic and genomic methods are readily available for follow up studies. A wealth of publicly available and well-curated data about the organism's physiology, biochemistry, gene expression, and ecology can be accessed to help in interpreting results. Experimental design can be conceptually simple. An ancestral population, usually started from a single colony, is propagated under selection in a controlled environment. A sample of the ancestor can be stored cryogenically for later comparisons with the evolved population. After a sufficient number of generations, the ancestral and evolved lines can be tested for differences in phenotype and genotype, and their relative fitness under specific conditions can be measured by head-to-head competition. Experiments can be designed to elucidate the dynamics of an advantageous mutation entering and spreading through a population. They can also be structured to identify and quantify beneficial mutations that increase the fitness of the organism in a specific niche. In the latter case, a powerful analytic approach is available: whole genome sequencing of evolved strains, followed by the construction of test strains that are isogenic except for individual mutations. The remainder of this review will focus on several studies with the *B. subtilis* system that illustrate the potential of this approach to analyze the adaptation of endospore-formers to their environment. Table 1 summarizes these studies, also giving mention to a few experiments that we have otherwise omitted from the discussion below for the sake of brevity. We will also ignore the longest-running and least controlled *B. subtilis* laboratory-evolution experiment–the domestication of lab strains–and focus instead on efforts designed to test the adaptive capacity of the Bacillus lifestyle.

Evolution with relaxed and stringent sporulation requirements

Because the endospore has played a critical role in *Bacillus* evolution, we examined the impact of stringent or relaxed selection for sporulation in the laboratory. Five batch cultures of a genetically marked *B. subtilis* strain were propagated in a complex liquid medium that induced sporulation (Sporulating, or S populations), and five cultures in a similar medium that repressed sporulation (Repressed, or R populations). At daily intervals we diluted the R populations 1:100 into fresh repressing medium, but subjected the S populations to heat shock to select for spores before diluting them into fresh sporulation medium (Maughan *et al*., 2006). We continued this regimen for 6000 generations, froze aliquots of each culture at 50-generation intervals, and tracked the evolution of these cultures using phenotypic tests along with population genetic and whole-genome techniques (Maughan and Nicholson, 2004; Maughan *et al*., 2006; Maughan *et al*., 2007; Maughan *et al*., 2009; Brown *et al*., 2011; Maughan and Nicholson, 2011; Nicholson, 2012).

Within 100–200 generations, both S and R populations developed a large diversity of colony morphotypes (which declined in later generations), and sporulation-defective colonies began arising in R populations. These observations suggested a rapid diversification, consistent with a rise in mutation rate. Fluctuation tests confirmed that indeed the mutation rate in the evolving populations was increasing by as much as 1–2 orders of magnitude (Maughan *et al*., 2006). The mechanism of this increase is likely similar to that underlying the well-studied phenomenon of stationary-phase mutagenesis in *B. subtilis* cells subjected to non-lethal stress (Robleto *et al*., 2007).

Competition experiments showed that over the course of their evolution, both S and R populations gained in fitness over the ancestral strain. Both populations generated diversity via an increase in mutation rate and evolved higher fitness in their environments by accumulating mutations that: (i) decreased the lag time for adjusting to fresh medium, (ii) increased their growth rates, and (iii) inactivated biosynthetic pathways superfluous in complex medium (Maughan *et al*., 2006). In R populations, mutations accumulated that inactivated sporulation, a trait no longer under active selection (Maughan *et al*., 2007). In nature, Bacillaceae deploy a host of functions to cope with a constantly changing environment, a concept known as phenotypic plasticity. DNA microarrays revealed that descendants from R populations in particular had lost plasticity in their ability to alter global transcription patterns in response to growth in the sporulation-inducing environment. This finding, coupled with the greater fitness of R populations in sporulation-repressing media, indicated that the populations were evolving from generalists into niche specialists (Maughan *et al*., 2009).

We tested for 'reductive evolution' or 'genomic erosion', i.e., loss of large dispensable regions of the genome, by probing DNA microarrays made from

Table 1. Examples of experimental evolution in *Bacillus subtilis*.

Selection	Evolved phenotypes	References
Domestication	Loss of biofilm and fruiting body formation, swarming motility and prophage content; increased competence	(Fajardo-Cavazos et al., 2016; Myagmarjav et al., 2016)
Soil microcosm	Increased population density	(Graham and Istock, 1979)
Stringent sporulation	Decreased lag-time, increased growth rate, loss of superfluous biosynthetic pathways	(Maughan and Nicholson, 2004; Maughan et al., 2006; 2007; 2009; Brown et al., 2011; Maughan and Nicholson, 2011; 2012)
Relaxed sporulation	As above, plus asporogeny, continued growth in postexponential phase, switch from acetoin to acetate production, small colonies, filamentation, nonmotility	As above
Ultraviolet radiation	Increased resistance to UV, X-rays, hydrogen peroxide	(Wassmann et al., 2010; 2011; 2012)
Liquid culture	Changes in colony morphology (~110 generations)	(Koeppel et al., 2013)
Low pressure	Increased fitness at low pressure (temperature-dependent)	(Waters et al., 2015)
High temperature	More regular cell morphology near upper limit; altered membrane lipids; deregulated heat shock regulon	(Zeigler, unpublished)

ancestral strain 168 with labelled DNA from evolved cultures (Maughan *et al*., 2009). No large deletions were detected in the S populations, but a large deletion of ~10 kbp was found in two R populations, resulting in a partial loss of an operon encoding the lipopeptide antibiotic plipastatin (Maughan *et al*., 2009); a similar deletion is known to have occurred in *B. subtilis* laboratory strain PY79 during its descent from ancestral strains (Zeigler *et al*., 2008). Thus, most of the mutations leading to adaptive phenotypes over 6,000 generations were not due to gross genomic losses, but to small changes, such as single nucleotide polymorphisms (SNPs) or small insertion/deletions (InDels).

About 1000–2000 generations into the experiment, an evolutionary sweep occurred (Maughan and Nicholson, 2011). In four of five R populations, a distinct small-colony morphotype arose and swept through the populations within a few hundred generations. The new variants grew to a higher cell density in sporulation-repressing medium and exhibited altered phenotypes such as filamentation and/or loss of motility. We isolated a strain (WN716) from the post-sweep event and compared it with an isolate (WN715) typical of the pre-sweep population. Competition experiments proved that post-sweep strain WN716 had indeed gained a large competitive advantage over both the ancestor and the pre-sweep strain WN715; in mixed culture, WN716 grew at a faster rate and continued to grow in postexponential phase, while WN715 dramatically lost viability during stationary phase. Transcriptome profiling showed that the operons encoding pyruvate dehydrogenase and purine and pyrimidine biosynthetic pathways were transcribed at higher levels in post-sweep strain WN716, together with numerous genes involved in adaptation to stress. The data were consistent with WN716's continued growth and its response to stress in the postexponential phase. There was a simultaneous decrease in transcription of operons encoding autolysins, flagella and chemotaxis functions, membrane-associated transporters and cytochromes, sporulation initiation, competence, extracellular enzymes, and antibiotic production. These data were consistent with the filamentous and nonmotile phenotype of strain WN716, as well as with a defect in its activation of transition-state functions in favour of continued growth in postexponential phase. In post-sweep strain WN716, transcription of the *alsSD* operon, responsible for acetoin fermentation, was among the most severely down-regulated. As a result, WN716 had switched its fermentative pattern from production of acetoin to acetate, with concomitant lowering of the medium pH from 7.0 to ~4.5. This observation suggested that acid stress was the environmental condition to which WN716 had evolved resistance, and which pre-sweep strain WN715 lacked. The increased fitness of WN716, then, was not due to a single mutation but to multiple changes resulting in its complex alteration of phenotypic traits (Maughan and Nicholson, 2011).

Pre-sweep strain WN715 and post-sweep strain WN716 were compared by genome sequence analysis (Brown *et al*., 2011). In the ~460 generations that elapsed between WN715 and WN716, a total of 34 SNPs and +1 insertions in coding regions of known annotated genes, and 11 SNPs in intergenic regions, had occurred. One nonsense mutation was predicted to inactivate the ECF sigma factor SigW, which controls a regulon involved in resistance to membrane-damaging agents and bacteriocins. Another mutation was found in *alsR*, a positive transcriptional regulator of acetoin fermentation (116). Although loss of motility in strain WN716 likely contributed to increased fitness, and multiple motility/chemotaxis operons were down-regulated,

we failed to find any mutation in the motility-specific sigma factor SigD (Brown *et al*., 2011; Maughan and Nicholson, 2011).

We directly tested whether inactivation of these pleiotropic regulators indeed contributed to the increased fitness of WN716 by constructing in the ancestral strain WN624 insertional knockouts of the *alsR, sigW*, and *sigD* genes, singly and in combination. The set of mutants were then tested in pairwise competition experiments vs. the ancestor (Nicholson, 2012). Each single knockout showed an incremental increase in fitness, and the triple *alsR, sigW, sigD* mutant displayed the greatest fitness increase compared to the ancestor (relative fitness of 1.130 ± 0.013). However, the triple mutant fell short of the dramatic increase displayed by strain WN716 (relative fitness of 1.322 ± 0.003). Therefore, this approach uncovered some, but not all, factors responsible for the WN716 fitness increase.

Evolution with selection for low-pressure growth

Earth's mean atmospheric pressure at sea level is ~101.3 kPa and ranges from ~25 kPa at the top of Mt. Everest to ~105.5 kPa at the Dead Sea. The atmospheric pressure on Mars is more than 100-fold lower than Earth's, averaging ~0.7 kPa and ranging from ~0.1 kPa at the top of Olympus Mons to ~1 kPa at the bottom of Hellas Basin (Fajardo-Cavazos *et al*., 2016). Growth of *B. subtilis* is inhibited at pressures below 2.5 kPa, 2–3 times above the highest pressure on the surface of Mars (Schuerger and Nicholson, 2006). Incubation of *B. subtilis* at low P (5 kPa) significantly altered the expression of 10 regulons, most notably an up-regulation of 86 transcripts of the General Stress Response (GSR) regulon (Waters *et al*., 2014). Transcription of GSR genes is controlled by RNA polymerase containing sigma-B ($Esig^B$), and expression of the GSR gene *ctc* was induced at low pressure in an $Esig^B$-dependent manner (Waters *et al*., 2014).

To elucidate the requirements of low-pressure growth, *B. subtilis* strain WN624 was propagated in rich liquid medium for 1000 generations at 27°C and 5 kPa, a pressure just above the minimum required for growth (Nicholson *et al*., 2010). Strain WN1106, isolated from the 1000-generation culture, out-competed the ancestral strain at 5 kPa, but not at Earth-normal pressure. Genome sequencing detected only 8 mutations in WN1106: SNPs in the *fliI*, *parC*, *resD*, *ytoI*, *yvlD*, *bacD* and *walK* genes, and an in-frame, 9-nucleotide deletion in the *rnjB* gene, *rnjBΔ9* (Waters *et al*., 2015). The *rnjB* gene encodes subunit RNase J2 of the RNA degradosome, an enzyme complex that governs global RNA turnover in *B. subtilis* (Cho, 2017). We constructed isogenic *B. subtilis* strains carrying either wild-type $rnjB^+$ or the *rnjBΔ9* deletion, and competed them at pressures of ~101.3 or 5 kPa and at temperatures of 20°, 25° or 30°C. Outcomes depended on the combination of T and P used. At 20°C the mutant strain was less fit than wild-type at both pressures, while at 30°C the mutant was more fit at both pressures. Only at 25°C (close to the T at which the evolution experiment was conducted) was the mutant more fit at low P and less fit at standard P (H. Nguyen and W.L. Nicholson, unpublished data)– highlighting the linkage between T and P in environmental research. Experiments are underway using RNA-seq technology to compare the wild-type and mutant transcriptomes under these conditions.

Evolution with selection for UV resistance

Similar concerns motivated researchers at the German Aerospace Center (DLR) to study the adaptation of *B. subtilis* to ultraviolet radiation with wavelengths of 200–400 nm (Wassmann *et al*., 2010; 2011; Wassmann *et al*., 2012). A comparable radiation environment exists on present-day Mars (Nicholson, 2009), and would have existed on Earth's surface during the Archean eon (4.0–2.5 Ga), when the endospore trait may have arisen. This directed evolution experiment allowed an ancestral population of *B. subtilis* to grow under non-sporulating conditions. Every tenth generation, the population was exposed to a pulse of polychromatic UV radiation. Four selection experiments were conducted in parallel, alongside a control that received no irradiation. After 69 cycles – about 700 generations – each evolved population, but not the control, exhibited a 4.5-fold increase in UV resistance relative to the ancestor. All four populations simultaneously evolved increased resistance to X-rays, and two populations exhibited enhanced resistance to hydrogen peroxide (Wassmann *et al*., 2010). Strain MW01, an isolate from one of the evolved lines, was tested for UV resistance at five points in its life cycle: lag, exponential, and stationary phases; as mature endospores; and during germination. MW01 showed higher resistance than the ancestor in all growth phases and during germination, and in spores exposed to high UV fluxes (Wassmann *et al*., 2011). In the subsequent ADAPT experiment, MW01 spores were tested in Low Earth Orbit, exposed either to full spectrum solar radiation and high vacuum or to simulated martian conditions, with attenuated ultraviolet light and a low-pressure CO_2 atmosphere. After 18 months the spores were returned for analysis. Unsurprisingly, spores exposed to full solar radiation were inactivated, while those with shielding showed moderate survival frequencies. The viability of spores exposed to simulated martian conditions was reduced 1–2 orders of magnitude, while viability of shielded spores was only reduced to 10–40% of initial levels. Pre-flight simulation

experiments in the laboratory, however, measured similar responses for MW01 and the ancestral strain, suggesting that the mechanisms of UV resistance in the evolved strain are primarily active in growing or stationary-phase cells, not in dormant spores (Wassmann *et al*., 2012).

Evolution with selection for high-temperature growth

Finally, recent experiments have used laboratory evolution to isolate *B. subtilis* strains adapted to high-temperature growth (D. R. Zeigler, unpublished). It is known that *E. coli* populations can adapt to a variety of temperature regimes, including temperatures near the growth maximum (Deatherage *et al*., 2017). Motivations for a similar adaptation of *B. subtilis* are three-fold. First, the topic of cell stress is a significant research front, in which *B. subtilis* is a commonly used system (Pane-Farre *et al*., 2017). Second, a *B. subtilis* strain adapted to high-temperature growth could have practical value for biotechnological applications, potentially reducing energy use and lowering production costs for fermentation, as has been noted for thermophilic platforms (Taylor *et al*., 2011). Finally, growth-temperature adaptation could play a significant role in the evolution of endospore-forming bacteria in novel environments.

The selection regime for these experiments involved an alternation of conditions: ten generations in a rich medium (Brain Heart Infusion) that does not support sporulation, followed by ten generations in a glucose-lactate-glutamate minimal medium (CDSM) in which spores form at high frequency. Every 20th generation, endospores were heat-selected before the cycle was repeated. For the first 500 generations, incubation was at 50°C in BHI and 48°C in CDSM, since experiments had determined these to be the maximum growth temperatures in each medium. For the final 500 generations, the temperatures were increased to 51°C and 49°C, respectively. This alternation was intended to favor the emergence of generalists adapted to high-temperature growth under both conditions, rather than specialists adapted to one but maladapted to the other. In this way it was hoped to identify key processes underlying survival at the upper end of the temperature range for *B. subtilis*.

After 1000 generations, two evolved lines were obtained with faster growth and more regular cell morphology at high temperatures than the ancestral strain. An isolate from one of the lines was analyzed by genome sequencing. Three mutations caused amino acid changes in proteins associated with the stress response: HrcA, a transcriptional repressor of heat-shock genes; FtsH, a heat-shock protein involved in cell division, sporulation, and cell envelope stress; and RelA, an alarmone synthetase involved in adaptation to various stress conditions. A fourth mutation was located in the promoter of *sigW*, a sigma factor that coordinates the transcriptional response to cell-envelope stress, while a fifth caused an amino acid change in RsiW, an anti-sigma factor that opposes the function of SigW. Another mutation affected MreB, a cell shape-determining protein involved in cell wall elongation. These results raise the possibility that cell envelope function and integrity are important limitations for high-temperature growth in *B. subtilis*. The selected strain showed a change in membrane fatty acid composition relative to the ancestor at moderate growth temperatures, with a marked decrease in 15:0 iso fatty acids and a corresponding increase in 15:0 and 17:0 anteiso fatty acids. Alteration of membrane lipid composition is a known function of SigW, and regulation of membrane fluidity is a universal strategy of cells in response to temperature changes (Helmann, 2016). Experiments are underway to determine which of these mutations may confer a fitness advantage to *B. subtilis* under high-temperature conditions.

Conclusion

We hope that these examples illustrate both the conceptual simplicity and analytical power of laboratory evolution to reveal the subtle genetic changes often responsible for bacterial adaptation to novel environmental niches. Further, we encourage others to use this approach to explore the mechanisms by which organisms with the Bacillus lifestyle have not only survived, but thrived on earth–and perhaps beyond.

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