The evolution of phenotypic diversification in heterogeneous microcosms maintained by a liquid-handling robot.

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Abstract
The survival of a population in a novel environment can depend upon the evolution of new traits—a process known as local adaptation. Such adaptation to a novel environment is often presumed to occur in tandem with a reduction in fitness in the ancestral environment. However, it is not known whether performance trade-offs develop more often in populations that experience only a homogeneous novel environment or populations that experience a heterogeneous environment composed of both ancestral and novel environments. It was hypothesized that the likelihood of the emergence of performance trade-offs was dependent upon the ecological and evolutionary histories of the evolved populations. To investigate this premise, I performed a meta-analysis of local adaptation in published studies of experimental evolution. This literature review revealed that performance trade-offs are most likely to evolve when the environment is homogeneous rather than heterogeneous. Furthermore, in heterogeneous environments, trade-offs were more likely to develop when the environment was spatially, rather than temporally heterogeneous. The literature review demonstrated that there were too few long-term spatially heterogeneous evolution experiments, so I designed a system that can be used to conduct this type of an experiment with the bacteriophage phi-6, with the aid of a liquid-handling robot. A multi-day evolution experiment was conducted successfully with the robot, indicating potential for using the robot to perform long-term evolution experiments. Overall, these findings demonstrate that performance trade-offs do not always emerge when populations adapt to novel
environments, calling into question an assumption that is frequently adopted in local adaptation studies.

**Introduction**

Resource heterogeneity—variation in the resources that are present in a population’s environment—has been shown to promote the phenotypic diversification of microorganisms and macroorganisms (Bono et al. 2017; Hedrick 1986; Kassen 2002). Phenotypic diversification is the evolution of disparate resource-use strategies within a species over the course of several generations of selection. This phenomenon can be observed following local adaptation to a novel resource. The resource heterogeneity that promotes phenotypic diversification takes two forms: temporal heterogeneity and spatial heterogeneity. In temporal heterogeneity, the resources that a population can access are regimented through time cycles (e.g. at time T=0, the environment of a population has resource A, and at T=1 the environment of the population has resource B). In spatial heterogeneity, all the resources that a population can utilize occur consistently in the environment; as such, divisions of the population may evolve to specialize on portions of the potentially available resources in the environment (Bono et al. 2017).

Performance trade-offs can emerge when a population adapts to a novel environment. There is a theoretical expectation that adaptation to a novel environment will result in specialization on the resources in the novel environment. Since the novel and ancestral environments differ considerably in form, the resource specializations that improve fitness in each environment will likely differ. Trade-offs are then expected to emerge when these resource specializations come into conflict (i.e. the specialization that provides high fitness in the ancestral environment also provides low fitness in the novel environment). This conflict in
phenotype would result from conflict in genotype, a phenomenon known as antagonistic pleiotropy (Kawecki and Ebert 2004). However, this genetic basis for trade-offs has been difficult to observe in natural populations (Anderson et al. 2011). Many evolution experiments have been conducted in an attempt to elucidate the difference between the expectation of antagonistic pleiotropy and the findings from natural populations. The role of trade-offs in resource specialization has also been questioned: trade-offs did not emerge in some experiments that imposed selection for adaptation to a novel environment (Kassen 2002 and Bono et al. 2017). Furthermore, it was not known if performance trade-offs were more likely to develop during adaptation to homogeneous or heterogeneous environments, when environmental heterogeneity was spatial or temporal, and during long-term or short-term evolution experiments (Bono et al. 2017).

The initial objective of this study was to review the local adaptation in experimental evolution literature, focusing on the experimental conditions under which trade-offs were most likely to develop. Through this literature review, I showed that performance trade-offs more often evolved in homogeneous rather than heterogeneous environments, when the heterogeneity was spatial rather than temporal, and when the experiment was conducted for a long time rather than a short time. Seeing that there were many fewer spatially heterogeneous experiments compared to temporally heterogeneous experiments (seven compared to fourteen, respectfully), I followed up on the literature review by designing and conducting spatially heterogeneous evolution experiments with \( \Phi 6 \). The literature review demonstrated that there are very few long-term evolution experiments, so I devised an experimental design that would enable the propagation of \( \Phi 6 \) populations for many hundreds of generations. A liquid-handling robot was used to conduct these experiments, as liquid-handling robots excel at consistently replicating
experimental procedures and enable round-the-clock propagation of phage populations. Through these experiments, I show that φ6 is a suitable system for studying the evolution of performance trade-offs in future long-term evolution experiments. In addition, I show that liquid-handling robots are capable of conducting evolution experiments continuously.

**Materials and Methods**

*Literature Review*

Forty experimental evolution studies were included in the literature review. The taxon (virus, bacteria, unicellular eukaryote), species, selective environment, number of generations, environmental variability (homogeneous/heterogeneous), heterogeneity type (temporal/spatial), and presence of trade-offs (yes/no/mixed) were recorded for each study. A trade-off was defined to be an evolved population’s reduced performance in an environment compared to their ancestral population’s performance in that environment. For instance, if all populations of *E. coli* which evolved in an environment with both acetate and glucose performed worse on acetate alone than their ancestor did, trade-offs were observed in that study. ‘Mixed’ meant that trade-offs were observed in some replicate evolved populations but not all. Significance testing for the effects of taxon, environmental variability, heterogeneity type, and experiment length on emergence of trade-offs were performed by my collaborators Lisa M. Bono and Christina L. Burch. See Bono et al. (2017) for more details on study selection and data analysis.

*φ6 and bacteria lineages*

The φ6 used in these experiments are descended from the Vidaver et al. isolate (1973). The ancestral population is derived from a single plaque, so all phages in the experiment are assumed to be clones; the ancestral population was stored at -20°C in 20% glycerol, 80% LC
media (liquid culture media made of 10 grams bacto-tryptone, 5 grams yeast extract, and 5 grams sodium chloride per liter of deionized water). The ancestral host of $\phi$6 is *Pseudomonas syringae* pathovar *phaseolicola* strain HB10Y (hereafter referred to as Pp). Ancestral $\phi$6 is able to infect Pp, but it cannot infect the novel host, *P. pseudoalcaligenes* pathovar ERA (hereafter referred to as ERA). A single base pair mutation can allow $\phi$6 to infect ERA (Duffy et al. 2006). Both host lineages originate from individual colonies that were cultured, centrifuged, resuspended in 60% glycerol/40% liquid culture media, and stored at -80°C. All $\phi$6 and host lines were provided by Christina Burch (University of North Carolina, Chapel Hill).

**Robot workstation**

A Beckman Coulter Biomek 2000 liquid handling robot was used to conduct the serial transfer experiments. The robot is capable of pipetting volumes as large as 1 mL and as small as 1 $\mu$L around the clock. Programming the robot is done via drag-and-drop command menus. Host cultures were incubated and phage infected hosts in wells of a 24-well round-bottomed plate. When serial transfers are conducted manually, cultures are incubated in a water bath shaker to keep the temperature constant at 25°C, to keep cells and phage well-mixed, and to aerate the cultures. To achieve these tasks on the robot, stirring of both host cultures and $\phi$6 evolution wells on the robot was performed using micro-stir plates and ‘flea’ sized stir bars (~2 mm in length). Stir bars (stir speed: 500 RPM) were used to aerate the host cultures and to distribute the phage evenly throughout the experimental well. These tasks are usually accomplished with a water bath shaker (shaker speed: 200 RPM) during manual transfers. The stir plates produced a significant amount of heat over time, raising the temperature of LC media in the 24-well plates to around 35°C within a few hours. Shallow trays of water, in combination with a stir bar placed
under the 24-well plate, diffused the heat created by the micro-stir plates so that evaporative cooling could keep the temperatures of LC media between 25 and 27°C (see Supplement 1 in the appendix for diagram of 24-well plate setup).

**Host propagation**

Host growth and φ6 transfer protocols were adapted from previously published φ6 local adaptation experiments (Bono et al. 2012; Bono et al. 2015; Bono et al. 2017). ERA and Pp liquid cultures were started by manually transferring a single colony of each host to a well filled with LC media in the 24-well plate on the robot (see Supplements 2 and 3 in the appendix for layouts of wells in 24-well plates). Following an overnight (approximately 30 hours) incubation with stirring on the robot, 50 µL of the resulting Pp culture and 1 or 5 µL of the resulting ERA culture were transferred to 2 mL LC media. These diluted cultures were incubated for 6 hours to produce exponentially growing cultures for the single-transfer experiments (Figure 1). This process was repeated to produce the exponential cultures for the multi-transfer experiments, with the only adjustment being that 75 µL of Pp and ERA overnight cultures were transferred to 3 mL of LC media to start exponential cultures for these experiments.
Figure 1. Robot transfer protocol for single transfer (LS-51 & LS-52) host range evolution experiments. Pp exponential cultures are made from a 1:40 Pp overnight : LC media dilution. A: ERA exponential is made from a 1:400 ERA overnight : LC media dilution. B: ERA exponential is made from a 1:2000 ERA overnight : LC media dilution.

Single transfer experiments

In the single transfer experiments (LS-51 and LS-52), 500 μL of the Pp and ERA exponential cultures were transferred to the experimental well (1 mL of LC is in the experimental well to start). Following this, φ6 was added to this experimental well to a density of 1.3*10^6 phage/mL (multiplicity of infection [MOI] is equal to 0.01, i.e. 1 phage for every 100 Pp cells). Phage were allowed to infect the hosts for six hours—about 3 φ6 generations. Then, the experimental well was filtered to exclude any bacteria, and the descendant φ6 were plated on a multi-host lawn to determine both the density of descendant φ6 in the experimental well and if any phage had evolved the ability to infect ERA. These plaque assays were conducted by mixing descendant φ6 with 200 μL of Pp overnight, 50 μL of ERA overnight, and 3 mL of 0.5% top agar (LC media with 0.5% agar), and pouring the mixture onto 1.5% bottom agar (LC
media with 1.5% agar) plates. When plated on a multi-host lawn, plaques of specialist ϕ6 should appear turbid, while plaques of generalist ϕ6 should appear clear. The descendant ϕ6 were stored at -20°C in 20% glycerol, 80% LC media.

Multiple transfer experiments

The multiple transfer experiments (LS-87, LS-89, and LS-90) were conducted similarly to the single transfer experiments, except that 5 transfers were completed instead of 1 (Figure 2). In LS-87 and LS-89, 500 μL of the Pp and ERA exponential cultures were transferred to the experimental well (2 mL of LC are in the experimental wells to start). Following this, ϕ6 was added to this experimental well to a density of 1.3*10^6 phage/mL (MOI = 0.01), as in the single transfer experiments. Phage were allowed to infect the hosts for six hours—about 3 ϕ6 generations. Then, 1 mL of a 10^-4 dilution of the 1st experimental well was transferred to the 2nd experimental well. Phage densities reached approximately 10^{10} phage/mL by the end of 6 hours of infection, so this 10^-4 dilution ensured that 10^6 phage/mL were present at the start of the 2nd transfer. Following the transfer of phage to the 2nd experimental well, 500 μL each of Pp and ERA exponential cultures were added. This process of incubation and transfer to a new well was performed until the 5th transfer and incubation was completed. The 5th transfer of descendant phage in LS-87 and LS-89 were plated exactly like the single transfer experiments. LS-87 and LS-89 isolates were stored at -20°C in 40% glycerol, 60% LC media.

The LS-90 multiple transfer experiment used the descendant phage from the LS-87 5th transfer as the ancestral phage in the experiment. Details of incubation, plating, and 5th transfer isolate storage of LS-90 phage are otherwise identical to those of LS-87 and LS-89.
**Figure 2.** Robot transfer protocol for multiple transfer (LS-87, LS-89, and LS-90) host range evolution experiments. Pp and ERA exponential cultures are made from a 1:40 overnight : LC media dilutions. Ancestral $\phi 6$ in LS-90 was descendant $\phi 6$ from 5th transfer of LS-87. Five transfer and incubation cycles were completed prior to plating descendant $\phi 6$.

*Exponential culture titering*

Cells in the single transfer exponential cultures in LS-51 and LS-52 and the 5th transfer exponential cultures in LS-89 and LS-90 were titered by diluting to $10^{-6}$ or $10^{-7}$ of the exponential culture volume in LC media, spreading the diluted volume on 1.5% bottom agar LC media plates with 5-10 sterile glass beads, and then counting the colonies on the plates. These colony counts were then divided by the plated dilution factor to obtain an estimate of the cell density in the exponential cultures (see Supplement 4 in the appendix for cell density estimates).
Results

Literature review

I reviewed a large collection of published evolution experiments to identify factors that affect the probability of observing trade-offs during local adaptation. Factors that were investigated included taxon, environmental variability, heterogeneity type, and experiment length. Trade-offs were significantly more likely to emerge during selection in a homogeneous environment than in a heterogeneous environment (Figure 3A, \( P = 0.0149, n = 40 \) studies). Only considering experiments conducted in heterogeneous environments, trade-offs were more likely to emerge when the heterogeneity was spatial rather than temporal (Figure 3B, \( P = 0.0298, n = 21 \) studies). Neither taxon nor experiment length had a significant effect (at the \( P < 0.05 \) level) on trade-offs emerging when all studies were included in the analysis. However, experiment length significantly increased the emergence of trade-offs for bacteria which evolved in a homogeneous environment (Figure 3C, \( P = 0.0158, n = 18 \) studies). Experiment length did not significantly affect the emergence of trade-offs in either viruses or unicellular eukaryotes during selection (\( P > 0.2 \) for both tests, \( n = 11 \) and 3 studies, respectively).
Figure 3. The emergence of performance trade-offs in laboratory evolution experiments depends on the environmental variability and the length of time selection is imposed. (A) Among the full set of experiments which compared the performance of evolved and ancestral populations in alternative environments, trade-offs were more often detected following selection in homogeneous than in heterogeneous environments. (B) Among the set of experiments conducted in heterogeneous environments, trade-offs were more often detected when the heterogeneity was spatial than when it was temporal. (C) Among the set of bacterial experiments conducted in homogeneous environments, trade-offs were more often detected in longer than in shorter experiments. In all panels, bars show the number of studies in which zero (trade-offs detected = no), some (trade-offs detected = mixed) or all (trade-offs detected = yes) of the replicate evolved populations exhibited worse performance than the ancestor used to found the experiment in at least one of the alternative experimental environments. Experiment length is represented by generations of selection: $10^1$ represents 10 to 99 generations, $10^2$ represents 100 to 999 generations, $10^3$ represents 1000 to 9999 generations, and $10^4$ represents 10000 to 99999 generations. Figure reproduced from Bono et al. (2017).
\(\phi 6\) experiments

In previous experiments (Bono et al. 2015; Bono et al. 2017), the Burch lab has used the bacteriophage \(\phi 6\) as a model organism to study phenotypic diversification in spatially heterogeneous environments. \(\phi 6\) is a pathogen of \textit{Pseudomonas} bacteria. Following infection of host bacteria, \(\phi 6\) has a replication time of approximately 100 minutes; infection by a single virion can yield 200-400 descendant phage (Chao et al. 1997). \(\phi 6\) has a very high genome-wide mutation rate (between 0.036 and 0.067 per generation) due to its RNA genome (Burch and Chao 2004). Within a multi-host environment, specialist \(\phi 6\) can evolve the ability to infect a novel host, becoming generalists. Consistent with the results of the literature review, generalist \(\phi 6\) grown in a single-host environment tend to evolve performance trade-offs on the alternative host, whereas generalist \(\phi 6\) grown in multi-host environments do not tend to evolve performance trade-offs during 300 generations of selection (Bono et al. 2017). However, the literature review also suggested that performance trade-offs are more likely to emerge in longer experiments. In the case of \(\phi 6\), 300 generations of selection in a spatially heterogeneous environment may not be enough time for performance trade-offs to evolve. \(\phi 6\) could be used as a model organism for studying long-term performance in a spatially heterogeneous environment, but the manual techniques that have been used in shorter evolution experiments could not be performed long-term; a 900 generation \(\phi 6\) evolution experiment would require 300 transfers performed by hand (adapting the design from Bono et al. 2017). This level of manual transferring is unsustainable, especially when multiple replications of the experiment are considered. However, robot experimenters can conduct evolution experiments continuously, potentially decreasing the amount of time required to conduct many generations long evolution experiments.
To that end, here I report the results of single and multi-transfer φ6 host-range evolution experiments conducted with a liquid-handling robot. Recall that every transfer cycle was initiated with $1.3 \times 10^6$ phage/mL. φ6 phages were able to infect, replicate within, and lyse Pp, their ancestral host in LS-51, LS-52, and LS-87 (Table 1). In these experiments, the φ6 population density increased by a factor of 100 to 1000 times that of the starting population.

During manual transfer, $10^{10}$ phage/mL is expected following 6 hours of incubation with host (see Bono et al. 2012 supplemental material), and descendant phage from LS-51 and LS-87 were close to this expected density. LS-89 and LS-90 had fewer viable phage after the 5th transfer.

<table>
<thead>
<tr>
<th>Name</th>
<th>Number of transfers</th>
<th>ERA exponential dilution treatment</th>
<th>Average concentration of descendant φ6 in evolution well (phage/mL)</th>
<th>Standard deviation of φ6 concentration (phage/mL)</th>
<th>Plaque turbidity</th>
</tr>
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<tbody>
<tr>
<td>LS-51</td>
<td>1</td>
<td>1:400</td>
<td>$6.54 \times 10^9$</td>
<td>$1.45 \times 10^9$</td>
<td>turbid</td>
</tr>
<tr>
<td>LS-52</td>
<td>1</td>
<td>1:2000</td>
<td>$2.66 \times 10^8$</td>
<td>$4.28 \times 10^7$</td>
<td>turbid</td>
</tr>
<tr>
<td>LS-87</td>
<td>5</td>
<td>1:40</td>
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<td>$6.42 \times 10^8$</td>
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</tr>
<tr>
<td>LS-89</td>
<td>5</td>
<td>1:40</td>
<td>&lt; 5.00 $\times 10^6$</td>
<td>--------------------</td>
<td>turbid</td>
</tr>
<tr>
<td>LS-90</td>
<td>5</td>
<td>1:40</td>
<td>&lt; 3.3 $\times 10^5$</td>
<td>--------------------</td>
<td>turbid</td>
</tr>
</tbody>
</table>

Table 1. Descendant φ6 concentrations and plaque appearance from evolution experiments. In multi-transfer experiments, phage concentration was determined after the 5th transfer.

Concentrations are averaged from plaque counts from φ6 platings (7 averaged plaque counts for LS-51, LS-52, and LS-87). A ‘<’ next to a value means there were too few plaques to give an estimate for average concentration of phage in the experimental well.
Though phage in LS-51, LS-52, and LS-87 replicated to the expected densities, no host-range mutants were observed (Figure 4). When plated on a lawn of the ancestral host (Pp) and novel host (ERA), plaques created by mutants which can infect both ERA and Pp will appear clear (see Figure 4-IV for clear plaque reference). All plaques from mixed host platings of LS-51, LS-52, and LS-87 were turbid, indicating that, if any phage had acquired the ability to infect ERA during these short evolution experiments, they were still present at frequencies below ~1/100.
**Figure 4.** Images of plaques developed by phage from evolution experiments. 

I. Phage from LS-51 plated on a mixed host lawn (Pp + ERA) produce turbid plaques. 

II. Phage from LS-52 plated on a mixed host lawn produce turbid plaques. 

III. Phage from 5th transfer of LS-87 plated on mixed host lawn produce turbid plaques. 

IV. Phage from LS-87 plated on a single host lawn (Pp only) produce clear plaques. Scale bar is 1 cm.
Discussion

Long-standing theory suggests adaptation to one environment will accompany maladaptation in an alternative environment (Kawecki and Ebert 2004). However, my review of the experimental evolution literature suggested that trade-offs do not always emerge during local adaptation. This was especially true when the selective environment was heterogeneous rather than homogeneous, and when that heterogeneity was temporal rather than spatial. These outcomes can be explained through selection for generalism. In an environment with resource heterogeneity, there is a selective advantage in utilizing all of the resources well. When only a single homogeneous resource is present, generalism cannot be selected for, as selection is blind to fitnesses on resources that are not present. Maladaptation on an alternative resource—through the acquisition of deleterious mutations—would be selected against in the heterogeneous experiments, but not the homogeneous experiments.

Similarly, trade-offs are less likely to emerge in the temporally heterogeneous experiments than the spatially heterogeneous experiments because the temporally heterogeneous experiments more strongly select for generalism. All resources will necessarily be experienced in temporally heterogeneous regimen, as the population is shifted from resource to resource at some time interval. In a spatially heterogeneous regimen, there is an equal chance of utilizing either resource. This equal chance will result in some members of the experimental population experiencing one resource less than the other. In these members, trade-offs will not be strongly selected against, since these members do not frequently utilize the resource on which they have reduced fitness.

Longer experiments were more likely to have trade-offs emerge, but this effect was limited to homogeneous experiments conducted with bacteria. That this trend was not observed
in viruses or unicellular eukaryotes does not strongly suggest that time does not have an effect on the emergence of performance trade-offs in these taxa. Almost all of the long-term evolution experiments were conducted with bacteria, and those few conducted for tens of thousands of generations were only performed with *E. coli*. There is a dearth of long-term evolution experiments, and even fewer that test for trade-offs in a descendant population compared to their ancestral population.

Liquid-handling robots could prove useful for conducting long-term evolution experiments. A major limiting factor in the conducting of long-term evolution experiments is the need for researchers to design the experiment around their work schedule. Robots can conduct experiments autonomously every hour of the day, so an experimental population can proceed through many more generations of replication in a single day than is seen when researchers conduct evolution experiments.

In recent years, liquid-handling robots have seen usage in a few microbial evolution experiments (Bell and Gonzalez 2009; Gonzalez and Bell 2012; Kerr et al. 2006; King et al. 2004), but none of these studies were concerned with the emergence of performance trade-offs following selection in disparate environments. That being the case, the ϕ6 experiments performed here represent a proof of concept on using robots to conduct this sort of evolution experiment, with a few caveats: 1. No generalist mutants were observed and 2. The experiments successfully conducted—meaning that there are indisputably more phage at the end of the experiment than at the initiation—on the robot are few in number. Addressing the first caveat, generalist mutants are known to arise within 4-10 transfers (Bono et al. 2012). A maximum of 5 transfers were performed in the successful evolution experiments, so the lack of generalist mutants could be explained by the stochasticity of mutations. Addressing the latter caveat, while
there are only 3 successful experiments with \( \Phi 6 \) transferred on the robot, these successful experiments demonstrate that \( \Phi 6 \) has the potential to replicate well on the robot system. At the outset of designing experimental procedures on the robot (months before those described here), having the robot pipet from one well to another without a catastrophic technical error was a minor miracle. In that case, achieving successful phage replication in three out of five experiments represents substantial progress. The absence of consistency concerning the descendant \( \Phi 6 \) concentrations remains an issue, but I feel it is not an insurmountable one.

The experimental evolution literature review demonstrated that trade-off emergence is not obligatory during evolution in homogeneous or heterogeneous environments. But the genetic causes of trade-off emergence are still not well understood. Disentangling this uncertainty requires future evolution experiments to incorporate diverse taxa over long timescales of selection. The preliminary \( \Phi 6 \) evolution results shown here demonstrate that in the future the conduct of long-term evolution experiments can be expedited by liquid-handling robots.

**Acknowledgements**

I would like to thank Christina L. Burch, Lisa M. Bono, Amy Maddox, Sonia Singhal, Grace Stafford, Falecia R. Metcalf, and Caitlin B. Moffatt for their assistance with this project. Christina L. Burch helped edit this work, assisted with the literature review, advised the design of the \( \Phi 6 \) experiments, and provided insights on every facet of this project. Lisa M. Bono also assisted with the literature review. Amy Maddox, Sonia Singhal, Falecia R. Metcalf, and Caitlin B. Moffat also helped edit this work. Grace Stafford helped build the LC plate photography rig.
References


**Appendix**

Supplement 1. Cross-sectional diagram of 24-well plate within a tray of water, all on top of a micro-stir plate. Triangular cutout in 24-well plate is to allow water flow underneath the plate; these were cut on all sides.
Supplement 2. Top-down diagram of 24-well single transfer (LS-51 and LS-52) evolution experiment plate. Overnight (O/N) and exponential (exp) host cultures start the experiment with 2 mL of LC media; φ6 experimental well starts with 1 mL of LC media, but by the end of the experiment the volume of this well increased to 2 mL following the transfer of 500 μL to this well from both exponential cultures.
**Supplement 3.** Top-down diagram of 24-well multi-transfer (LS-87, LS-89, and LS-90) evolution experiment plates. Overnight (O/N) and exponential (exp) host cultures start the experiment with 3 mL of LC media; 6 experimental wells start with 2 mL of LC media, but the volume of these wells increased to 3 mL following the transfer of 500 μL to these wells from Pp and ERA exponential cultures. Dilution wells contain 3 mL of LC media. Stir bars were used to mix LC media.
<table>
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<tr>
<th>Experiment</th>
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<th>Standard deviation of host concentration (cells/mL)</th>
<th>Plates counted (n)</th>
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**Supplement 4.** Cell concentrations for exponential cultures used in the single and multi-transfer evolution experiments. Cell concentrations for the multi-transfer experiments were from the 5<sup>th</sup> transfer exponential cultures. Host exponential cultures were not titered for LS-87.