Repeatability of the adaptation

of *Pseudomonas fluorescens* to low glucose

by Oleksiy Teselkin

uOttawa

THESIS SUBMITTED TO THE
FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF MASTER OF SCIENCE
DEPARTMENT OF BIOLOGY
UNIVERSITY OF OTTAWA

To Miss YSJ

Acknowledgements

Many of life's failures are people who did not realize how close they were to success when they gave up.
–Edison

I would like to thank all the talented colleagues who I had the privilege to work with and who made me realize that the journey matters as much as the destination.

Risking to plagiarize Susan Bailey (thanks for the first plate-pouring lessons, Sue!), I will thank Aaron Hinz for his patient expertise (and expert patience) in troubleshooting everything from media to dilutions to primers and not letting me forget that the order matters. Nothing in this project would not have been possible without him.
I wish to thank Susanne Kraemer, Gabriel Perron and Nicolas Rode for the helpful discussions on data analysis.
A big thank you to my committee members, Stéphane Aris-Brosou, Linda Bonen, and Andrew Simons, for their valuable advice and time they take to read these lines.
I am also grateful to all the members of the Kassen and Wong labs for their feedback on the improvements to the presentation of my project.
Thanks to Gabriel Perron and Nicolas Rode for translating the abstract and filling the lab with energetic music.
I am most indebted, however, to my advisor, Rees Kassen, for taking me onboard and supporting throughout my time in Ottawa, blazing fast online and offline responses, setting a high standard of excellence, pushing me, believing in me, giving the advice one needs but does not necessarily wants to hear, and most importantly, for helping to unleash my inner quester.

Laus illi debetur et a me gratia maior.

Oleksiy Teselkin
Ottawa, spring 2014

Declaration of own

I declare that the thesis which I am submitting to the University of Ottawa for the degree Master of Science has not been submitted by me to any other university for degree purposes, and I am aware that, should the thesis be accepted, I must submit additional copies as required by the relevant regulations.

SIGNATURE:	Oleksiy Teselkin	DATE:	28.IV-2014

Abstract

Inspired by Gould, who claimed life would be arriving at a different outcome each time it were allowed to run from the same beginning, I have attempted to determine the repeatability of the adaptive course of one *Pseudomonas fluorescens* lineage. In addition, my study aimed to establish whether the likelihood of parallel evolution of the two synonymous single-nucleotide substitutions was contingent upon a prior motility-impairing deletion or a prior increase in fitness. Further, the study was designed to provide empirical data addressing the long-standing question of the effect of starting fitness on the ensuing rate of adaptation.

Although no exact replay of the initial evolutionary trajectory was observed, I have demonstrated that *gtsB,* but not *gtsC* gene*,* is likely to be a mutational hotspot under the low glucose with a recovery of two undescribed mutations in *gtsB*. My data are consistent with a notion that substitutions in *gtsB* may be contingent upon Δ_{35kB}(*fliJ*-PFLU4466) motility-impairing deletion, but not the fitness increase associated with it.

Finally, the features of the adaptive landscape of *P. fluorescens* in the minimal glucose provide languid support for Fisher's hypothesis of a decrease in adaptation rate with the rise in the starting fitness.

Taken together, these original results reinforce the non-negligible role of history in shaping the outcomes of biological evolution and call for caution in attempting a formulation of rigid predictive models of evolutionary change.

Résumé

Inspiré par les travaux de Stephen J. Gould qui affirmait que la vie sur terre arriverait à une forme différente si elle repartait à zéro, je présente ici mes travaux où je teste la reproductibilité du cours adaptatif d'une lignée expérimentale de *Pseudomonas fluorescens*. L'objectif de cette étude était de déterminer si la probabilité que deux mutations synonymes évoluent en parallèle est affectée par la présence d'une délétion affectant la motilité de la bactérie ou de l'augmentation de la valeur sélective de celle-ci. De plus, le design expérimental de cette étude permet de tester si la valeur sélective initiale d'une population affecte le taux d'adaptation de cette même population.

Bien d'une reproductibilité exacte du cours adaptatif initial ne fut pas observée, je démontre que le gène *gtsB* est probablement un « hotspot » mutationnel permettant l'adaptation à de bas niveau de glucose, ayant trouvé deux mutations dans ce site; alors que le gène *gtsC* ne l'est pas. Mes données sont également conséquentes avec le fait que les mutation dans le gène *gtsB* dépendent de l'effet de la délétion Δ_{35kB}(*fliJ*-PFLU4466) affectant la motilité de la bactérie, mais non de l'augmentation de la valeur sélective qui y est associée. Finalement, la forme du plateau adaptative associé à de bas niveaux de glucose chez *P. fluorescens* supporte l'hypothèse émise par Fisher qui stipule que le taux d'adaptation d'un organisme diminue avec la valeur sélective initiale qui y est associée.

L'ensemble de ces résultats supporte le rôle non-négligeable de l'histoire de vie d'une population en ce qui attrait à l'évolution future de cette même population. Aussi, ces résultats appelle à la prudence quand vient le temps de formuler des modèles prédictifs des changements évolutifs d'une population.

Contents

List of tables

List of figures

1 Introduction

Biology is a study, not in being, but in becoming.
–Carl Woese

There is no place for dogma in science.
–J. Robert Oppenheimer

What is it that distinguishes science from other human endeavors? According to Carl Popper, it is not the empirical method of science — astrology would qualify then — but rather the inherent falsifiability of scientific claims. What is unfalsifiable would therefore be unscientific, enabling a demarcation criterion: predictions of astrology are, for example, not amenable to disproof.

Does Darwinism fall within the circumference of genuine science? Popper claimed that the position of universal laws is necessary for a candidate enterprise to fall within the realm of genuine science [1]. The study of evolution, according to him, is of a historical, singular nature, and therefore unable to produce any testable predictions. The science of evolution has often lacked in its predictive capacity, being more about explanations, identifying the causes of past events, rather than predicting the future. Trying to explain the historical course of evolutionary events by looking at fossils adds little to testing the theory of natural selection. Popper proceeds that he "has come to the conclusion that Darwinism is not a testable scientific theory, but a metaphysical research program — a possible framework for testable scientific theories." [2] This statement casts a long shadow on all of biology,

as its “intuitively obvious” essence lies “not in things as they are, but in things coming into existence.” [3]

The three components of a Darwinian system are random variation, differential propensities for contribution to future generations and a mechanism of inheritance linking the two [4]. Can natural selection account for adaptation? And can its predictions be empirically tested in a quantitative way?

Microbial model systems, known since the times of Darwin [5], but largely forgotten until the late 1980s, brought with them the promise of introducing the strict experimental method into a previously descriptive discipline and securing a firmer position of evolutionary biology in the domain of *bona fide* science.
The major advantage of microbial laboratory evolution lies in the degree of replication and environmental control it offers [6].
The scale of microbial experiments is essentially unlimited. This is aided by such features of microorganisms as their large population sizes, short generation times, and high mutation rates. [7, 8] Short generation times allow for a quick turnover of individuals, enabling the more adapted lineages to expand and the beneficial mutations to sweep through the population. Large population sizes minimize the effect of genetic drift and increase the likelihood of the emergence of rare beneficial mutations of large effect due to high mutation supply rates (the product of population size and mutation rate). While the per-base rates of spontaneous mutation have generally been believed not to differ systematically across major domains of life [9, 10], it has been reported that bacteria may display elevated per genome adaptive mutation rates [11]. As such, bacterial models make it easier to

study rare beneficial mutations, further enhancing the microbial utility for empirical evolutionary research.

The ultimate advantage of microbial experimental models is the predictive empiricism of fitness measures that they enable. Fitness is measured by the ability to compete for resources. In competition experiments, this would equate to a rate at which focal strains displace a common competitor. With reproductive success (offspring quantity) justified as a measure of genetic contribution to future generations [6, 12], a direct competition assay is feasible. This experimental integral fitness measure stands in sharp contrast to the retrospective (and often arbitrary) trait-by-trait comparative method that evolutionary biologists classically rely on to allow for making of quantitative predictions on the direction of evolutionary change. Moreover, competitive fitness measure, whereby evolvants directly compete with their immediate or distant ancestors evolved in the same environment, can also serve as a proxy measure for adaptedness.

Rev. Dr. William Dallinger, the author of the first published microbial evolution experiment, demonstrated a remarkable increase in the temperature tolerance of protozoans during a long-term cultivation in a prototype 'chemostat' of his own invention. [5] This old and venerable work, while missing replication and end-point relative fitness measurements, both indispensable to modern research, received some praise from Darwin. Dallinger's work is also notable for providing a demonstration of correlated fitness trade-offs, since high-temperature adapted lineages lost their ability to thrive at the lower base temperature where they started

— a frequent phenomenon exemplifying the relative nature of evolutionary progress.

Even as adaptation to a certain condition is frequently associated with correlated costs of maladaptation to other environments, can there still be a direct path to the most favorable adaptive solution under given circumstances? Or are evolutionary trajectories historically unique as future evolution is largely determined by random mutation supply despite being constricted by the common foundation of prior genomic architecture? In an attempt to answer these questions, I bring the explanatory potential of the lawfulness of natural selection into present work in the context of strengthening the predictive capacity of the evolutionary research [13, 14].

The motivation of Dallinger has been to demonstrate experimentally "whether it is possible by change of environment . . . . to superinduce changes of an adaptive character, if the observations extend over a sufficiently long period." [15] The current study takes this further by aiming to investigate whether it is possible for the *same* environment to superinduce the *same* (or at least similar) changes of an adaptive character in *multiple independent isogenic populations*. In addition, this work has been inspired by a desire to explore the question of evolutionary contingency — do later adaptive changes depend on the preceding ones, and how will starting from different successive points along the adaptive route affect the character and timing of the resulting outcomes?

I focused on correlating the fitness and genetic measures of adaptation in this study. Addressing what is spread in-between the blueprint and its manifestation,

the mechanistic physiology linking the genotype and phenotype, was not an objective of this research and will not be addressed in the present body of work.

In what follows below I develop hypotheses and — in a true Popperian fashion — falsify their predictions against the evidence.

2 Replaying life's tape, *or*
A review of the historical contingency in the adaptive process

The first thing that strikes one's eye on our planet is the diversity of living forms on it. The major factors of evolutionary change — variability, inheritance and selection — are believed to be responsible for the branching progression of life from the first lucky protocell to the entire modern world.

> "The affinities of all the beings of the same class have sometimes been represented by a great tree. [..] The green and budding twigs may represent existing species; and those produced during former years may represent the long succession of extinct species. At each period of growth all the growing twigs have tried to branch out on all sides, and to overtop and kill the surrounding twigs and branches, in the same manner as species and groups of species have at all times overmastered other species in the great battle for life," — Darwin [16]

While the grand-scale evolutionary scheme is generally believed to have followed the *divergent* pattern of trait evolution [17-20], despite the overwhelming plethora of documented instances of unexpected trait *convergence* [21], it is to a third classic evolutionary trend of *parallelism* that this review will be devoted.

Parallel evolution refers to the independent evolution of the same trait in closely related lineages [22, 23]. It is commonly believed that this results from the contribution of natural selection, although it has been suggested that it may also be the biased production of the initial genetic variation in related clades that is responsible for phenotypic parallelism [24, 25]. Some of the most striking evidence

for parallelism comes from rigorous evolution studies such as [26-31], where examples of parallel phenotypic evolution underlain by parallel genomic evolution are described. Sticklebacks, for instance, repeatedly evolved morphological changes in the separate natural populations, strongly arguing for the adaptive value of these changes [31].

It needs to be mentioned that a molecular biologist's definition of parallelism, though clearly related to the above phenotypic definition, from which it derives, is more specific and precise. Parallel changes to macromolecular sequences are construed as involving a transition from the same ancestral state to the same derived state, as contrasted with convergent changes — from differing ancestral states to a shared derived state [32]. Following Wood and colleagues [32], I will adopt the following (more flexible) version of the parallelism definition for the operational purpose of this work: parallel genotypic adaptation as the independent evolution of homologous loci to fulfill the functionally equivalent role in two or more lineages.

If we separate historical clonal populations each into a number of identical replicates and let these accrue chance genetic variation in a given environment, any resulting resemblance among them must be caused by natural selection, despite differing history and the vagaries of chance [14]. With artificial selection experiments thus controlling for both the effects of history and chance in elementary laboratory environments, one may wonder whether there exists any resolute adaptive outcome determined for the initially-identical clonal replicates and underlain by similar genetic pathways. This is the evolutionary contingency

question, a subject that has been receiving abundant attention from evolutionists and philosophers alike, oftentimes only to further complicate and obfuscate the discourse [33].

Capacity and necessity: antecedents of parallel evolution The debate on the relative importance of genetic accidents and selective opportunities in determining the outcomes of evolutionary processes has persisted since the dawn of modern evolutionary thinking. The importance of chance in shaping the adaptive process has often been underscored in both classic [34] and recent [35] literature. Given the absence of unequivocal evidence for the existence of directed variation [36-38, but see 39] — by which I mean the causation between environmental changes and fitness-increasing variations facilitated by these changes, i.e. variation "in a fitting manner," as Darwin himself put it [40] — the process of mutagenesis is apparently random. Its outcomes are constantly sieved by natural selection, which tolerates the useful and liquidates the maladaptive.

With chance and necessity "jointly intricated in the stuff of life" [41], an interesting question arises: What would happen should we gain the ability to rewind the tape of life, and let it run again and again? As put by Stephen Jay Gould:

> "I call this experiment "replaying life's tape." You press the rewind button and, making sure you thoroughly erase everything that actually happened, go back to any time and place in the past. . . . Then let the tape run again and see if the repetition looks at all like the original"[42].

Gould would expect us to arrive at a different living world each time we attempted such a replay. "*Any* [emphasis mine—OT] replay of the tape would lead evolution down a pathway radically different from the road actually taken," — he wrote.

(Ibid.) “If you wish to understand patterns of long historical sequences, pray for randomness.” [43]

Evolution is a very special process. In order to witness Gould’s replay, a time machine would be necessary. For lack of such a machine, the construct of macroevolution remains irreducible — neither a pure theory nor a fact. Instead, it is a collection of paleontological facts and the logical deductive inference drawn from it. The modern science paradigm, however, underscores the importance of falsifiable experiments in giving a shape to a scientific worldview [44]. That chance variation results in unpredictability of evolution is a hypothesis in need of testing. The recent rise of the laboratory-controlled microevolution studies of microbial populations, with their large population sizes, short generations and high mutation supply, makes possible the design of replicated, manipulative evolutionary experiments [45]. Should the same evolutionary outcome be guaranteed only if there were some direction in the process of variation itself or should evolution be predictable solely on the basis of selective considerations? It has been argued that because it is a matter of chance which variations arise, it is also a matter of chance which variations will be selected and hence which adaptive outcomes will obtain [46]. It would therefore follow that selection does not play an exclusive role in directing evolution. It has been observed, however, that evolutionarily relevant mutations are not distributed randomly across all genetic regions [47]. Pleiotropy, epistasis, phenotypic plasticity, strength of selection, and population history have all been documented as factors influencing the distribution of adaptive mutations [47]. A pleiotropic mutation, which influences several ostensibly unconnected traits, and

an epistatic mutation, the effect of which is modulated by other loci, can both limit the rate of simultaneous evolution across multiple traits because an optimization in one trait as a result of a mutation in a pleiotropic or epistatic gene will likely be linked to a misfit in other connected phenotypic dimensions, making the change disadvantageous as a whole. It is therefore to be expected that non-interacting mutations and pathways would often be favored in the course of the adaptive process, leading to high parallelism as a result of contingency [48-50]. Similarly, potential future long-term plasticity, the ability of a genotype to express alternative phenotypes which may come at considerable energetic costs, may often be traded for the short-term increased reproduction benefits often associated with reduced plasticity, thus providing another route for parallelism in evolution [51-53]. Elements of selection and population history can have dramatic effects on biasing the supply and assortment of evolutionarily relevant mutations, sometimes culminating in strikingly ordered parallel adaptations, e.g. [54-57, but see 58].

How predictable are the genetic underpinnings of phenotypic evolution? It has been argued that the 'protein tape of life' is generally more reproducible and predictable than its genetic counterpart, which is less constrained as to the variations it can accommodate [59]. As motivation of any scientific field progresses along the sequence of description, explanation, prediction, and, finally, intervention, how close are we to predicting a future state of population based on our knowledge of its present state and our control of the selection regimen applied? It has been reasoned that laboratory experiments allow one to clearly separate the effects of history, chance, and necessity in research microcosms [14]. One can begin by growing stocks

of the historically different populations from single cells and splitting these stocks into a desired number of the initially-indistinguishable replicates, thus assuring that populations of each type share the same heritage. If these populations were now propagated for an extended period of time under the indistinguishable environmental circumstances, any emergent resemblance among them must surely be ascribed to the methodical non-random perpetuation of the successful types harboring adaptive combinations of random variation. Fitness itself typically changes in these 'laboratory natural selection' experiments in the most predictable fashion, while the physiological and especially genetic bases of adaptation are proving more susceptible to historical canalization and chance diversion [14]. It is important to note that laboratory natural selection is distinct from other types of laboratory evolution such as artificial selection [60] as the role of the experimenter introducing her bias into the selection process is eliminated in the former case, bringing one's experimental design as close to nature as it gets in the controlled laboratory setting.

How satisfactory can the explanatory power of the above pan-selectionist rationale be, the rationale that evolutionary results can be forecasted based on the selective pressures only? Gould, interestingly, enunciated a complementary vision of contingency in his *Wonderful Life*:

> "I am not speaking of randomness…, but of the central principle of all history — contingency…. A historical explanation on an unpredictable sequence of antecedent states, where any major change in any step of the sequence would have altered the final result. This final result is therefore dependent, or contingent, upon everything that came before: the unerasable and determining signature of history" [42].

This passage complements the insufficiency perspective on contingency, quoted in an earlier passage of Gould's. Here, Gould articulates a vision of replaying life's tape now *not* over and over from the same setting, but with the initial conditions ever somewhat changed. It is worth emphasizing, however, how Gould's videotape analogy applies to both versions of contingency: contingency-unpredictability (though arguably not -indeterminacy) — as for an unwatched re-play, and contingency-determinacy — for a watched one.

Gould's notion of contingency as a chief explanation of organic history stands in opposition to Popper's probabilistic notion of natural law. The two possess different truth values. The former "after-the-fact" explanations may satisfy the *verifiability* criterion of scientific character, whereas the derived predictions of the latter may entertain being proved true or false — thus conforming to the *falsifiability* criterion of scientific character — an indisputably superior alternative, at least according to Popper [61]. Gould used the terms 'historical explanation' and 'historical narrative' arguably referring to causal dependence and unpredictability of evolution, respectively [62]. In his replay argument, with the complementary accounts of contingency in mind, Gould would reject the exclusive role of natural selection in shaping the adaptive outcomes, not questioning at the same time the predestinarianism of this outcome. What would appear to an observer as unintelligible results would by no means imply their randomness. While Gould did not offer an exhaustive account as to the possible nature of contingent factors having a large influence on the evolutionary outcomes, it has been speculated for a long time that these factors may resultant of the irreducible properties of the

Biosphere at large [21], specifically the volume of horizontal gene transfer as a creative factor in directing evolution, pervasive in natural systems [63-64, with some remarkable examples in 65-66]. While the evolutionary implications of these compelling new data in the face of an old idea remain to be assessed, a critical part of such an evaluation should be the question of limits of the scientific method as it applies to the study of organic evolution, which brings us back to what Karl Popper referred to as the poverty of historicism, or a lack of falsifiable predictive power in the study of evolution [67]. The classical approach of extracting the quantitative parameters of present natural systems to infer their *past*, such as in [68], falls short of meeting the Popperian notion of the genuine scientific character and his logic of the scientific process. One may therefore be justified in resorting to future *prediction* instead — at this stage with artificial experimental systems only. These systems may lack in the inferential capacity of the comparative/historical tradition in their ambition to simulate selection in the wild [69], but would allow for direct testing of predictions through refutations [70].

Faced with an uneasy conundrum of balancing empiricism and verisimilitude, I limit myself to setting a test to Gould's contingency hypothesis in a controlled microevolutionary laboratory experiment. Gould formulated his thesis in a classic Popperian way, discussing the impossibility of any — *any* — exact replay of the life's tape. While a number of studies have been inspired by him if not expressly undertaken to dissect his evolutionary proposition on whether history ever repeats itself [71-82], their results have been often contradictory and raised the question of generalizability, which has been confounding their interpretations. In addition, the level of experimental contingency was often found to vary depending on the

character in question, making it difficult to count the evidence 'for' and 'against'. Following the seminal work of Michael Travisano [77, 78], then in the Lenski group, I will set to falsify in what follows the applicability of Gould's thesis to a case of laboratory-evolved beneficial bacterial single nucleotide substitutions, to a review of which I will now proceed.

Synonymous mutations: genetics of adaptation. Evolutionary adaptation concerns itself with the dynamics of the emergence and fixation of beneficial mutations, and much of what we now know about adaptation owes to the abundant data from microbial evolution experiments of the recent decades [83, 84]. Adaptation has been defined as time-dependent shifting of the mean population phenotype to the one that best fits the current environment [85]. The study of quantitative genetics has been marked by the pervasive influence of Ronald Fisher's geometric analogy, which envisions populations progressively moving toward a fitness optimum — a combination of traits best matching an organism to its environment — through a sequential substitution of beneficial mutations. Fisher's pioneering work provided an elegant mathematical construction accounting for both the classical Darwinian micromutational gradualism and the rediscovered Mendelian laws of heredity [86] and later forming the foundation of the infinitesimal model of adaptation [87]. Fisher argued for decreasing contribution of larger effect mutations as populations are getting closer to a given phenotypic optimum on their adaptive path. The rationale he put forward was that a large shift in a phenotypic character, even in the right direction, would likely disrupt a complex system by overshooting the imaginary sphere around the optimal character value.

Fisher also clarified this idea with his microscope analogy, a large shift in any of microscope's parameters being unlikely to achieve the focus — a fitness optimum. A corollary to this would be an expectation for adaptation to comprise a series of mutations of decreasing magnitude [14]. This may mean that exceedingly rare beneficial mutations of largest effect would be responsible for dramatic initial fitness gains upon the start of adaptation in an unfamiliar environment, giving way for more frequent mutations of lesser effect to fine-tune the adaptation within the sub-optimal zone — a scenario which has been substantiated theoretically [88] and since received some empirical support [89-93]. It may be initially surprising to accept that early adaptation is governed by the improbable draws of exceptional magnitude, until one fully comprehends the genealogical view on the selectable lineages, lineages which amplify the information on fitter individuals in an exponential fashion. The nature of self-replicating nucleic acids, where types with greater replication rates invariably increase in frequency, gives rise to the emergent competitive exclusion principle: in a limiting environment one type will eventually prevail — a subtle but a truly powerful idea, the implications of which reach to the bottom of what is happening in the Darwinian systems [94]. The priority substitution of larger-effect mutations on the way toward the environmental optimum also gives rise to *diminishing returns epistasis*, whereby the marginal effect of each successive mutation decreases with the passage of time and increasing fitness [95], resulting in the quick decrease of the predicted rate of adaptation with the rise in the starting fitness. This phenomenon is often referred to as *negative epistasis for fitness*. Finally, increased genetic parallelism may obtain as fitter genotypes are theoreticized to suffer from lower beneficial mutation supply rate as

moving higher on a fitness ladder results in fewer remaining adaptive solutions [96]. For a given beneficial mutation, this theoretically develops into the increasing likelihood of fixation when in the fitter genetic backgrounds. Another bearing of Fisherian micromutationalism has been its support for the pan-selectionist perspective on the contingency debate [97], as minute nearly-continuous variation would easily lend itself to molding of the parallel phenotypic outcomes, determined solely by selection. Fisher's view would therefore largely oppose that of Gould, as fine-graining of variation would reduce the unpredictability of adaptation and diminish the role of any historical narrative in evolution, at least phenotypically if not at the DNA sequence level [98].

Beneficial mutations themselves, and especially their fitness effects, have been underresearched by population geneticists for a long time [99]. The distribution of fitness effects among the very common and easily observable deleterious mutations was what early studies concerned themselves with [e.g., 100-103]. Any discussion on how the frequency of beneficial mutations is orchestrated by natural selection needs to be preceded by an examination of the nature of these beneficial mutations, the question of how they emerge in the first place [104].

As evolutionary interest moved from the deleterious to the neutral class of mutations and the neutral theory of molecular evolution rose in prominence, most substitutions at the DNA-sequence level started being assumed to have no appreciable effect on fitness [105-107]. Such obvious classically neutral gene changes as third codon base substitutions — without a corresponding amino acid change due to degeneracy of the genetic code — were often cited in support of the

neutral theory of molecular evolution [108]. Can any genome changes happen without affecting phenotype at all? While neutral theory itself has often been brought into question in the recent decades [109-112], this became particularly true for synonymous mutations lending support to it [113]. Synonymous substitutions occur when a base in an exon of a protein-coding gene is substituted without resulting in a change of the encoded protein sequence. It has been tempting to think of them as silent in regard to their influence on phenotype, and by extension — fitness, but new results have opened various avenues for synonymous mutations to matter under the magnifying glass of selection, be it through their effect on protein folding [114, 115], mRNA folding [116], mRNA splicing and stability [117, 118], codon bias [119-121], or other mechanisms. In a similar vein, the recent intriguing report of Bailey and colleagues [122], which motivated me to conceive of the present study, reported the isolation of two synonymous single nucleotide substitutions that have sequentially evolved an experimental population of *Pseudomonas fluorescens*. The long-term persistence of mutations in lineages is moderated by selection [123]. Bailey and colleagues have reconstructed genetic and fitness dynamics of the events leading to and following the evolution of these substitutions. In addition, they engineered and competed the isogenic constructs of the substitutions in a gain-of-function experiment to prove their beneficiality. The mechanism of this beneficiality put forward involved enhanced expression of the gene to which they mapped [122]. While studies have previously documented the intermittent beneficiality of some synonymous mutations [e.g., 124-126], the study by Bailey and colleagues was the first of its kind to demonstrate the natural (or as natural as its gets in the laboratory) and spontaneous evolution of synonymous substitutions. This isolated

observation immediately raised the question of parallelism: how repeatable the evolution of Bailey's synonymous mutation will be, should the number of independent replicate populations be increased?

3 Repeatability of *Pseudomonas fluorescens* adaptation to low glucose

Prior work. There is little quantitative experimental evidence concerning the beneficial fitness effects of synonymous substitutions. They are often assumed to be selectively neutral or subject to weak negative selection. As far as I am aware, the recent work by Bailey and colleagues was among the first to produce firm experimental evidence for the possible adaptive value of synonymous substitutions [122]. These mutations evolved within the context of a larger experiment investigating the effect of ecological opportunity on the evolutionary dynamics of bacterial populations. An initially isogenic population of *Pseudomonas fluorescens* SBW25 was propagated for 1000 generations by daily transfers in a minimal glucose batch culture. Relative fitness — as measured by the descendant population in head-to-head competitions with its isogenic ancestor — increased significantly by 14.79% (t-test, P=.02) relative to the ancestor by generation 1000 [122]. Whole-genome sequencing of generation 1000 populations revealed two synonymous single nucleotide substitutions in the PFLU4845 gene (A15A: GC**A**→GC**G** and G38G: GG**C**→GG**T**) as well as a 35kB (Δ_{35kB} *fliJ*-PFLU4466) motility-impairing deletion, all absent in the motile ancestor. PFLU4845 gene has been putatively annotated as *glucose transport subunit (gts)B*, an uncharacterized component in the glucose ABC cassette transport system [127]. As samples were frozen every 200-generations in

the course of experiment, it was possible to estimate the timing of key evolutionary events by isolating and targeted (*gtsB*-targeted) sequencing of sets of 24 colonies from each 200-generation adaptive walk.

It has been discovered that the deletion (Δ_{35kB}) appeared around generation 200 and was nearly replaced by Δ_{35kB}+A15A genotype by generation 800 only to be largely replaced again by Δ_{35kB}+G38G genotype by generation 1000 (Figure 1). The observed substitution dynamics were in accord with strong periodic selection acting on advantageous alleles [123]. The resultant loss of diversity in the population can be taken as another testament to the strength of the selective pressure behind the sweeps of the new genotypes. Assaying competitive fitness of Δ_{35kB}+A15A and Δ_{35kB}+G38G genotypes against the ancestor was undertaken to quantify the fitness effects of these genetic changes. Additionally, two allelic replacement strains containing the synonymous mutations of interest (A15A and G15G) but not having the Δ_{35kB} deletion (and thus not present in the original experimental populations) were constructed to further confirm the beneficial effect of synonymous mutations as well as their relative fitness, with G15G predicted fitter than A15A. Finally, a naturally evolved Δ_{35kB} alone was also competed against the ancestor. The results of these competitions revealed that Δ_{35kB}+G38G confers a 13.07% (±1.64% s.e.) and Δ_{35kB}+A15A a 10.76% (±1.51% s.e.) of fitness advantage over the wild-type *P. fluorescens* SBW25 (S. Bailey, personal communication). It has been estimated that the deletion alone increases fitness by 9±3% s.e. Finally, in case of the constructed strains, G38G gives a 8.73%(±.79% s.e.) and A15A gives a 7.25% (±.55% s.e.) fitness increase [122]. Taken together, these results provide a strong evidence for the real effect of synonymous substitutions and raise a suggestion of negative epistatic

interaction ('diminishing returns') between each of the mutations and the Δ_{35kB} deletion in the glucose environment — a phenomenon previously described in many experimental systems [128-130]. In the present case, each of the synonymous substitutions in the presence of the deletion leads to a fitness increase lesser than the one reasonably expected from their solitary presence in the constructed isogenic strains.

It has been emphasized by Bailey and colleagues that the evolved physiological effects of the synonymous substitutions are almost certainly specific to the minimal glucose environment [122] — in contrast, for instance, to the motility-impairing deletion, which is likely to represent an example of general adaptation to the shaken culture, saving the organisms the energy cost of the superfluous operations, and thus optimizing the efficiency of resource allocation. The strong glucose specificity claim of Bailey and colleagues is grounded on several lines of converging evidence:

1. No fitness benefit on alternative carbon sources such as mannose and xylose;
2. Decrease in fitness on glucose in loss-of-function *gtsB*-knockout strains (-14.23%±1.42% s.e., $p<.0001$, compared to the wild type), but not on mannose and xylose ($p=.96$) [122];
3. Isolation of a third beneficial (but non-synonymous) mutation (A10T) in the same general (5') region of *gtsB* and occurrence of all three mutations (A10T, A15A, G38G) in glucose only.
4. Prior research inference in regard to the possible function of *gtsB:* PFLU4845 was predicted to code for a permease subunit of a glucose-

dependent ABC transporter [127, 131-134] and so would be expected to be glucose-specific in its activity.

Purpose of study and research questions. Gould would call the above intriguing adaptation story an example of a historical explanation. It is crucial to note that the described sequence of events:

$$(\text{wild-type} \rightarrow \Delta_{35kB}\ \text{deletion} \rightarrow \Delta_{35kB} + \text{A15A} \rightarrow \Delta_{35kB} + \text{G38G})$$

took place in only one population out of three maintained in the minimal glucose environment. Following Gould, one may ask whether we would get the same evolutionary outcome on multiple replays if started with the same lineages in the same environments. And also whether the mutational order (i.e., the deletion preceding the substitution of the synonymous mutations) would make for a big difference in evolutionary outcomes among independent replays.

These two principal questions will put to a test the combined notion of evolutionary contingency as defined by Gould in his final work:

> "the tendency of complex systems [..] to be unpredictable in principle from full knowledge of antecedent conditions, but fully explainable after time's actual unfoldings" [135].

Specifically, I ask the following questions:

1. How frequently do the same synonymous substitutions arise when evolution is allowed to run again?
2. Would the synonymous substitutions arise more frequently in the deletion strain?

3. Would the synonymous substitutions arise more frequently in a strain with comparable to Δ_{35kB} starting fitness but an unrelated genetic change? I will from now on refer to this strain of nearly equivalent fitness to the deletion strain ($t(5)=-1.1011$, $p=.32$) as the *G3* background. A combination of (2) and (3) would allow me to distinguish between the effects of fitness and genotype on the parallel evolution of the synonymous substitutions.
4. Finally, I also wish to know whether the rate of fitness increase is dependent upon the starting fitness across all genotypes. This would be a test of Fisher's geometric model.

Hypotheses and predictions. I develop the following hypotheses:

1. Large genomic deletions increase the mutation rate at nearby sites, leading to an increased probability of observing nearby mutations and increased rate of parallel evolution of these mutations.
2. Higher starting fitness leads to an increased probability of parallelism and to a reduced rate of fitness increase, both due to the lower availability of beneficial mutations once some of them have been already substituted.

I aim to test the following predictions:

1. a. Synonymous mutations are more likely to be seen evolving repeatedly in the background of Δ_{35kB} deletion than in a strain of comparable fitness but without the deletion (*G3*).

   b. Synonymous mutations will evolve in ⅓ of Δ_{35kB} deletion strain lineages.
2. a. Synonymous mutations are more likely to be seen evolving repeatedly in the *G3* background than in the wild-type strain.

b. The genotype with the lowest initial fitness (the wild-type) will show a greater fitness increase compared to the genotypes with higher starting fitness (Δ_{35kB} and *G3*).

Predictions (1a) and (2a) together define the relative ranking of expected parallelism, from the lowest in the wild-type to the highest in the Δ_{35kB} strain and allow one to distinguish between the effects of fitness and genotype on the parallel evolution of synonymous mutations. Prediction (1b) complements a combination of predictions (1a) and (2a) by specifying a quantitative expectation stemming from prior research, where the synonymous mutations occurred after Δ_{35kB} in 1 of 3 independent populations [122]. Finally, prediction (2b) is a direct test of Fisher's geometric model.

Materials and methods. *P. fluorescens* is ubiquitous in soil and water, flagellated (motile), plant-colonizing, rod-shaped, aerobic Gram-negative bacterium. It became a model organism in microbial ecology research owing to its environmental abundance, natural diversity and spectacular adaptive radiations that it repeatedly undergoes in the defined laboratory conditions [136]. The strain used in the present study (SBW25) originates from a 1989 isolation from a beet leaf (*Beta vulgaris*) in England and has not undergone any adaptation to laboratory conditions, with stocks having been maintained at -80°C over more than two decades [137]. The SBW25 genome is approximately 7 million bp in length [138]. The start of the experiment involved reviving *lacZ*-engineered SBW25, SWB25Δ_{35kB} and *G3* strains in 2 mL of a 9.57 mg/L minimal glucose medium (see Appendix A) for 24 hours and colony-plating of the liquid cultures. The experiment

commenced with starting a liquid culture of each founder strain from single colonies and subsequent 48-hour acclimation in a daily-transfer batch culture. The culture was daily diluted by transferring 20μL (or a million individuals) from old growth into a 2mL of fresh medium, a ratio of 1/100. This allowed for 6.64 ($2^{6.64}$=100) bacterial generations per growth cycle. The acclimated isogenic cultures of the three strains were then divided each into 24 replicates, organized into 4 columns (A through D) and 6 rows (1 thorough 6) on a microwell plate and numbered therefore A1 through D6. The strains continued to be maintained for 560 generations in the minimal glucose selection batch culture. A separate disposable 24-well plate for each starting genotype was used, giving a total of three plates. Plates were covered in foil, taped to a shaker and incubated at 28°C and 150rpm. The transfer regimen was kept at 20μL of bacterial culture into 2mL of fresh medium every 24 hours throughout the experiment. Bi-weekly, samples of each line were frozen in eppendorf tubes with 80% glycerol and plated on X-gal plates to screen for contamination. The 200-generation frozen fossil record provided for contamination avoidance and is available for revival and analysis. Another contamination safeguard was the positive-confirmation 'blue-white' screen made possible by using *lac*-marked strains for all evolving lines and X-gal medium plating (Appendix A). Lac-marking derives its name from the lactose operon of *Escherichia coli*. Lac-operon introduction has a useful property of turning lac-expressing cells blue on incubation with a lactose analog, X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside). Contaminants are unlikely to harbor lac-operon and will usually remain whitish, hence the name of the 'blue-white' screen.

An integral part of a selection experiment is the quantitative measurement of adaptation. It is important to keep in mind the distinction between the following two metrics of adaptation: adaptedness and fitness [14]. The former informs one about population *viability* and is measured by an increase in growth (i.e, optical density) of a pure culture. The latter informs one about population *competitiveness* and is measured by relative growth in a mixed culture.

Given:

1. That an increase in one measure would usually lead to a corresponding increase in the other one,
2. The ecological realism of mixed cultures,
3. The greater sensitivity of the fitness assay: the possibility of detection of even weak selection, through a mean change in strain frequency across the replicate competitions [14] —

I chose the relative competitive fitness assay as a proxy of adaptation in this work. Competitive fitness was calculated as

$$\omega=[(F_f/f_f)/(F_i/f_i)]^{(1/doublings)},$$

where *F* stands for the strain of interest, *f* for the SBW25 competitor and the subscripts refer to the *i*nitial and *f*inal frequencies — before and after a competition respectively. *Doublings* refer to the number of doublings, or generations, that occur between the initial and final plate counts. The discrimination between the two competing strains and their counting relied on the use of the *lacZ* marker for all of the evolved lines, as opposed to the unmarked wild type, the common competitor. The competing strains were mixed in equal ratios in the liquid culture, allowed to compete for 24h, after which the initial fitness was taken by plating a sample out

and counting the number of the resultant blue and white (wild-type tester) colonies. The second measurement took place another 24h after the first one.

Genotyping was implemented by two independent PCR amplifications of candidate genes for each sample and direct (i.e., without vector-mediated cloning) sequencing of the PCR products (McGill University Nanuq Center, Montreal). No step of DNA isolation or purification was undertaken. The primers used and the extent of coverage are given in the Figure 2. Alignment of the *gtsB* and *gtsC* genes sequencing data was to the *P. fluorescens* SBW25 public reference genome NC_012660 [138]. Genomic context of the location of these genes is shown in Figure 2 [139]. A full coverage of the neighboring genic loci of *gtsC* and *gtsB* was achieved (approximately 2 kB), with the prime locus of interest — the putative hot spot for both synonymous and nonsynonymous substitutions — having a double coverage with two different sequencing primers, *gtsB*_M13F and PFLU4845M13_F (Table 1, Figure 2).

Results. *Demonstration of adaptation by natural selection and of significantly greater adaptation rate of SBW25.*—Generation 560 competitive fitness data for each evolved population and the founders is given in Table 2 and plotted on figure 3. Across all populations, fitness has increased by a mean of 7.17% for the deletion background, 8.53% for *G3*, and 10.80% for the wild-type SBW25 (Figure 4). An analysis of variance showed that rate of fitness increase across the wild type, Δ_{35kB}, and *G3* genotypes was highly significantly different, $F(2, 68) = 8.739$, $p = .0004$. Post hoc analysis using Tukey's multiple comparisons test indicated that the average fitness increase was significantly greater for SBW25 lineages as compared to Δ_{35kB}

lineages (mean difference M = .0418, 95% c.i. = 0.0179 to 0.0658) than in the other two comparisons (q(68)=5.912, adjusted p= .00). In case of SBW25 compared to G3, the effect approached statistical significance (q(68)=3.064, adjusted p= .09).

Selection of two non-synonymous mutations in the deletion background only.—Generation 560 population-wide genotyping for the adjacent *gtsC* and *gtsB* loci has uncovered two mutations in *gtsB* in the two of the Δ_{35kB} populations (see Table 1):

- **C**5324094(32)**T** in B1
- **G**5324013(112)**T** in D2.

This corresponds to the following codon and amino acid changes, making both mutations non-synonymous:

- S11L {T**C**G>T**T**G} in B1
- G38C {**G**GC->**T**GC} in D2.

Both of the mutations have been confirmed by two independent PCR amplifications with different primers.

Colonies have been isolated and their sequencing undertaken to assess the relative frequency of S11L and G38C mutations, the latter of which appeared to be fixed based on a single chromatogram peak, whereas the former displayed a double peak on the chromatogram, suggesting a partial substitution within the sample population (Figure 5).

The colony PCR followed by sequencing corroborated these results, with 12 out of 12 D2 colonies testing positive for G38C, and 17 out of 28 (61%) of B1 colonies confirming S11L.

Further demonstration that gtsB *but not* gtsC *is a target of selection* .—Despite the similarity in the location, regulation, and function of the two genes, both of which are the members of the same operon encoding symmetrical permease subunits of an ABC glucose transporter (Figure 6), only the upstream region of *gtsB* has been shown to attract both synonymous [122] and non-synonymous mutations. The present work confirms this finding by mapping the newly described naturally evolved S11L and G38C mutations to *gtsB*, in addition to A10T, A15A, and G38G mutations previously described [122].

Δ_{35kB} *may exhibit a parallel evolution rate of 33% of changes to* gtsB.— Fisher's exact test was chosen for the analysis as some of both observed and expected values in the contingency examination were less than 10 at 1 degree of freedom. Two-tailed probability was used throughout as neither the previous research, nor physical limitations, nor did common sense suggest a direction of difference to make the use of a one-tailed P justified. A Fisher's exact test was performed to determine whether prediction (1b) that generated the expected value of ⅓ were supported by the data. I fail to reject the null hypothesis of independence (and by extension the underlying hypothesis of ⅓ parallel evolution from the deletion founder) based on the obtained two-tailed P value of .0723. A post hoc power analysis was performed to estimate achieved statistical power, based on pilot data from [122], where the substitutions in question evolved in a single population out of the three exposed to the minimal glucose environment. With an alpha = .05, expected parallelism rate of .33 and observed parallelism rate of .08, total sample size of 48 and allocation ratio of 1, the power (1 - beta error probability) achieved was .49.

The data have a tendency to conform to the predicted relative ranking of parallelism potential of the founder genotypes, but the association does not reach statistical significance.— The Freeman-Halton extension of Fisher's exact probability test was chosen to analyze the 2x3 contingency table of the genetic outcomes with non-directional probabilities values — for the same reasons as in the previous paragraph. I fail to reject the null hypothesis of independence (and by extension the underlying hypothesis of no difference in the evolution potential of the synonymous mutations among SBW25, Δ_{35kB}, and G3 genotypes) based on the obtained two-tailed P value of .3239.

Demonstration of no cross-contamination among the evolvants of different starting genotypes.—Generation 560 population genotypes have been redundantly confirmed with a combination of three approaches: sequencing, gel electrophoresis, and semi-solid agar motility screen. Δ_{35kB} deletion, not present in the other two genotypes, was positively confirmed through PCR and electrophoresis of the newly created by junction region (Figure 7). The populations of all three groups have been subjected to a semi-solid agar motility screen, confirming all of the Δ_{35kB} and G3 evolvants as non-motile (the ancestral state), and the majority of the SBW25 evolvants as motile (Figure 8). Finally, sequencing of a genetic marker contained within the Δ_{35kB} deletion and thus characteristic of G3 and SBW25 was undertaken, positively confirming the identity of all their populations.

Discussion. Our ability to propagate parallel isogenic populations of organisms with short generations under identical conditions enabled me to produce the much needed experimental evidence concerning the question of whether the evolution

from the same starting point and in the same selective environment can lead to different outcomes. This study challenged bacterial glucose uptake mechanism in a glucose-limited environment. It has been hypothesized that the synonymous single nucleotide substitutions will evolve in a third of lines started from SBW25 based on the prior investigation [122], where this happened in a single population out of the three exposed to the minimal glucose environment. As the substitutions in question were known to evolve in the SWB25Δ_{35kB} background only, the 35kB motility-eliminating deletion (located ~400 kB away from the putative hotspot of the synonymous mutations) has been expected to form a prerequisite for further evolution of A15A and G38G — a situation not unsimilar to the famous Lenski's citrate use innovation [55], where the emergence of citrate-utilizing variants invariably had to be preceded by a potentiating duplication. As Bailey's experiment was aimed at a different purpose, it did not involve a sufficient parallel replication of independent lines in the minimal glucose environment. This has been addressed in the present work, with 24x replication of each of the investigated genotypes — SWB25, Δ_{35kB}, and G3. The lack of the nucleotide-level parallel evolution (owing to the miss of any of the purported genetic changes) does not appear extremely surprising as the degree of parallelism is generally thought to decrease from fitness and phenotype to the exact same genes to the exact same nucleotide changes [59]. This was the conclusion of the important pioneering studies on the predictability of evolution, which suggested that evolutionary outcomes tend to be more similar in terms of the achieved fitness, but are often quite different with respect to the underlying genetics [77, 78], and this conclusion is largely supported by the experimental study of mine. Two independent PCR amplification products helped

to ensure that the recovered mutations were not PCR artifacts, but rather genuinely present in the original population templates. With multiple possible solutions generally available for any adaptive problem, what one may find surprising is the recovery of the G38C substitution, in the exact same codon as the previously reported G38G synonymous substitution. The data were found not inconsistent with the contingency-on-deletion hypothesis (1) and its ⅓ evolution rate prediction (1b), although the statistical significance of the effect vanished on the relative ranking comparison. In addition, with the achieved statistical power of .49, my experimental design of 24 replicates (1 24-well microplate) for each of the starting genotypes (based on logistic considerations) proved underpowered for the main objective of this study. It is definitely worth doubling the sample size for future research of my system to achieve more adequate power in excess of .8 or even triple it for a power of .96.

My study succeeded in falsifying the contingency-on-fitness hypothesis (2), thus *not* supporting Fisher's idea that the likelihood of fixation of any given mutation increases when in fitter genetic backgrounds, at least not for the synonymous substitutions in my experimental system. Interestingly, the Δ_{35kB}D2 lineage achieved a fixation of G38C and appears to exhibit a greater fitness compared to the mean of fellow Δ_{35kB} parallel lines (though the effect does not quite reach statistical significance, t(3)=1.8239, p=.17). The other recovered mutation, S11L in Δ_{35kB}B1, substituted for more than half of the population that it occurred and was selected within. Δ_{35kB}B1 reaches statistical significance of its fitness difference from the mean (t(3)=4.3886, p=.02, an increase of .0887 above the deletion lineages mean). On

aggregate, it is tempting to conjecture that S11L, being "more beneficial" than G38C (See Table 2 for competitive fitness data on the carriers of these mutations), has arisen later and not yet achieved fixation. The speculative nature of this argument should be kept in mind, however, as full genome sequencing has not been undertaken on any of generation 560 evolvants, and mutations elsewhere may be responsible for the observed fitness effect. Should full genome sequencing fail to identify changes elsewhere in B1 and D2, the order-of-appearance prediction could be put to a test with targeted sequencing of the frozen fossil record to reconstruct the population dynamics.

My results are generally in-line with Gould's perspective on evolutionary contingency. No exact replay has been observed, although the Δ_{35kB} deletion appeared to exert a non-negligible potentiating effect on mutations in the *gtsB* gene, whether synonymous ones or not. My work shows that the establishment of the role of synonymous mutations in adaptive evolution may require a much larger scale of independent lineage replication. Despite the fact that the present research concentrated on investigating the repeatability of the two synonymous substitutions only, it may be interesting to additionally probe the repeatability of the Δ_{35kB} deletion. Although the loss of motility is a common energy-saving adaptation to a shaken culture (and was observed among a convincing fraction of SBW25 populations, see Figure 8), one would not expect the exact same genetic change among a large fraction of possible disruptions in the flagella genes to be repeatedly responsible for it.

Another suggestion for future research is to address the possible issue of cross-contamination within parallel population of same genotypes with introduction of unique short nucleotide tags into each replicate population. While my approach relied on maintaining the proper technique at all times during the batch culture passaging, with additional confirmation by the absence of any suspicious patterns during the genome analysis (such as exactly same mutations in populations from adjacent wells), an introduction of extra controls may be a worthy investment for projects of longer duration. It needs to be kept in mind, however, that the selective neutrality of unique nucleotide tags will have to be proven in each case. In addition, manipulations required for such a step may disrupt the initial isogenicity of replicate populations by introducing genetic changes outside the desired selection regimen.

If *E.coli* were to serve as a guide, the *P. fluorescens* adaptive landscape in glucose should be less rugged and to show more of a propensity for parallel evolution, compared to the alternative carbon sources such as mannitol, fructose, or maltose [140]. This tendency would be in line with the other reported findings of the same organism in the same environment [141], a study that established both phenotypic outcomes and the underlying genetic routes less variable in glucose as opposed to xylose. Following these results, it may be interesting to research whether the Δ_{35kB} deletion would have a potentiating effect on the likelihood of the evolution of the glucose transporter genes in nutrients, utilizing the same transport pathway as glucose. Examples of these include mannitol, fructose, and mannose [140].

The data collected do bear some support to Fisher's expectation of *a decrease in the rate of adaptation with the rise in starting fitness* (2b). My results permit the conclusion that low starting fitness may have an effect on the subsequent adaptation rate. Specifically, the obtained evidence suggests that SBW25, which started at a lower fitness, showed a greater tendency for adaptation at the border of statistical significance. However, it should be noted that the starting fitness level must be low in order to see an effect. The intermediate fitness level of Δ_{35kB} demonstrated, in fact, the lowest fitness increase. The genotype that started with the lowest initial fitness (SBW25) showed the greatest increase in fitness, though the expected order does not hold for the middle (Δ_{35kB}) and the middle-high starter (G3). As the effect was only bordering on significance, at my level of replication I find it prudent to reserve a judgment on whether the rate of improvement indeed decelerates over time. By way of suggestion for the follow-up research, it would be useful to include more replicates of more starting genotypes, in particular a fitness-matched pair for SBW25. This would allow for a more rigorous statistical treatment of the issue.

An unexpected occurrence in the course of the population passaging of all genotypes has been the repeated emergence of a cryptic slow-growing phenotype (Figure 9). As populations have been re-started from a prior frozen stock in such cases, the phenomenon has not been investigated is sufficient detail and is definitely worth of more attention. Of note, a phenomenon of the emergence of non-culturable cells in the same organism has been described previously [142] and a possible mechanism of it has been recently proposed [143, 144]. This mechanism involves the transfer of integrative and conjugative elements, with transfer competence being a terminal

state. The remarkable natural diversity of Pseudomonad genome architecture may largely rely on acquisition of genetic information through recombination, a factor, the full extent of which may be irreducible to laboratory evolution [145]. In contrast, repeated sampling of clones identical at multiple loci at different locations and times have been taken as evidence for the pervasive clonality and low level of recombination in Pseudomonads [146, 147]. In light of these reports, an alternative and perhaps more likely hypothesis on the origin of the cryptic cells would involve a emergence of morphological diversity through cross-feeding [148, 149], a phenomenon previously undescribed in *P. fluorescence* on minimal glucose.

The powerful and well-controlled extent of laboratory microbial experimentation is not without its critics: the validity of projection of microbial findings beyond the exact setting of their occurrence has been doubted [150]. As a partial remedy, an *in situ* genetic survey of distantly sampled free-living Pseudomonads for the described substitutions may be undertaken to complement the laboratory approach. As some organic or amino acids are the preferred carbon sources in Pseudomonads [151], it may be argued that soil isolates of *P. fluorescens* sampled distantly from the plant rhizosphere, with which Pseudomonads are closely associated, would be more likely to sustain a selective pressure to optimize glucose intake. Extensive parallel evolution, even at the nucleotide level, with conservation of the majority of the genome has recently been reported in natural Pseudomonad isolates, a finding consistent with positive selection on accessory genes that facilitate environmental adaptation [152]. This reinforces the validity of the above proposal aiming to try

extrapolating the fundamentals of microbial evolution into the field to bridge the gap between a test tube and nature.

4 Conclusions

Laboratory experimental evolution complements the comparative method by increasing predictive capacity of evolutionary science.

The value of present research lies in that it recovers two previously undescribed mutations in the *gtsB* gene and establishes that the 5'-region of *gtsB,* but not *gtsC,* a possible adaptive hotspot in the low glucose. In addition, this study fails to disprove the idea that substitution in *gtsB* is contingent upon Δ_{35kB}(*fliJ*-PFLU4466) motility-impairing deletion.

Fisher's hypothesis that an increasing likelihood of fixation of any given mutation is expected in the fitter genetic backgrounds is not supported by gathered evidence. This work fails to reject, however, Fisher's hypothesis that a quick decrease of rate of adaptation is expected with the rise in the starting fitness.

Future studies should direct attention to both evolutionary and physiological agents behind the substitution-proneness of the 5'-region of *gtsB*. In addition, future research commands more extensive (and expensive) experimental designs. For instance, inclusion of a pair-matching genotype for SBW25 and greater replication of all founder genotypes.

This line of research is pivotal to both securing a firmer grounding of evolution as a predictive and empirical science, and developing scientific methodology and public policies aimed at securing health, environment and food supply in the face of increasing bacterial antibiotic resistance (e.g, *Pseudomonas aeruginosa*) and the growing world population.

Tables and figures

TABLE 1
Detected mutations

Sample	Sequencing primer name	Mutation	Codon change	Amino acid change
Populations:				
Δ_{35kB}B1	PFLU4845_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
Δ_{35kB}B1	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
Δ_{35kB}D2	PFLU4845_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C
Δ_{35kB}D2	*gtsB*_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C
Colony isolates:				
B1colony1	*gtsB*_M13F	-	-	-
B1colony2	*gtsB*_M13F	-	-	-
B1colony3	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony4	*gtsB*_M13F	**-**	-	-
B1colony5	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony6	*gtsB*_M13F	**-**	-	-
B1colony7	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony8	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony9	*gtsB*_M13F	**-**	-	-
B1colony10	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony11	*gtsB*_M13F	**-**	-	-
B1colony12	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony13	*gtsB*_M13F	**-**	-	-
B1colony14	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony15	*gtsB*_M13F	**-**	-	-
B1colony16	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony17	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony18	*gtsB*_M13F	**-**	-	-
B1colony19	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony20	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony21	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony22	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony23	*gtsB*_M13F	**-**	-	-
B1colony24	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony25	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony26	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony27	*gtsB*_M13F	**C**5324094(32)**T**	T**C**G>T**T**G	S11L
B1colony28	*gtsB*_M13F	**-**	-	-
D2colony1	*gtsB*_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C
D2colony2	*gtsB*_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C

D2colony3	*gtsB*_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C
D2colony4	*gtsB*_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C
D2colony5	*gtsB*_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C
D2colony6	*gtsB*_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C
D2colony7	*gtsB*_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C
D2colony8	*gtsB*_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C
D2colony9	*gtsB*_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C
D2colony10	*gtsB*_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C
D2colony11	*gtsB*_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C
D2colony12	*gtsB*_M13F	**G**5324013(112)**T**	**G**GC->**T**GC	G38C

TABLE 2
Competitive fitness

Population genotype	Mean fitness	Standard error of mean
<u>Ancestors:</u>	1.0853	.0129
Δ_{35kB}	1.1118	.0203
SBW25	1.010	.0089
G3	8	
<u>Evolved replicate populations:</u>		
Δ_{35kB}A1		
Δ_{35kB}A2		.0148
Δ_{35kB}A3	1.1814	.0199
Δ_{35kB}A4	1.1708	.0132
Δ_{35kB}A5	1.1259	.0225
Δ_{35kB}A6	1.1584	.0040
Δ_{35kB}B1	1.1472	.0168
Δ_{35kB}B2	1.1593	.0202
Δ_{35kB}B3	1.2457	.0155
Δ_{35kB}B4	1.2212	.0278
Δ_{35kB}B5	1.1339	.0054
Δ_{35kB}B6	1.1360	.0099
Δ_{35kB}C1	1.1148	.0131
Δ_{35kB}C2	1.1179	.0209
Δ_{35kB}C3	1.0850	.0261
Δ_{35kB}C4	1.2094	.0208
Δ_{35kB}C5	1.1060	.0173
Δ_{35kB}C6	1.1276	.0094
Δ_{35kB}D1	1.1455	.0124
Δ_{35kB}D2	1.1241	.0143
Δ_{35kB}D3	1.1526	.0205
Δ_{35kB}D4	1.1977	.0066
Δ_{35kB}D5	1.1795	.0290
Δ_{35kB}D6	1.1526	.0264
SBW25A1	1.1624	.0179
SBW25A2	1.2119	.0033
SBW25A3	1.2179	.0127
SBW25A4	1.1736	.0138
SBW25A5	1.1924	.0181
SBW25A6	1.1963	.0229
SBW25B1	1.1860	.0122
SBW25B2	1.2371	.0217
SBW25B3	1.2257	.0150
SBW25B4	1.1962	.0226
SBW25B5	1.2107	.0167
SBW25B6	1.2177	.082
SBW25C1	1.1803	.0198

SBW25C2	1.2599	.0223
SBW25C3	1.2686	.0180
SBW25C4	1.2095	.0268
SBW25C5	1.1791	.0299
SBW25C6	1.2056	.0194
SBW25D1	1.1638	.0169
SBW25D2	1.2143	.0056
SBW25D3	1.1363	.0313
SBW25D4	1.1667	.0261
SBW25D5	1.1112	.0203
SBW25D6	1.1854	.0237
G3A1	1.1731	.0103
G3A2	1.2228	ND
G3A3	ND	.0062
G3A4	1.1314	.0076
G3A5	1.1202	.0172
G3A6	1.1111	.0075
G3B1	1.1023	.0089
G3B2	1.1137	.0171
G3B3	1.1064	.0110
G3B4	1.0933	.0157
G3B5	1.0592	.0197
G3B6	1.1011	.0088
G3C1	1.1337	.0155
G3C2	1.0792	.0049
G3C3	1.1666	.0109
G3C4	1.1599	.0247
G3C5	1.1058	.0150
G3C6	1.1411	.0267
G3D1	1.1093	.0229
G3D2	1.1318	.0139
G3D3	1.1112	.0125
G3D4	1.1189	.0219
G3D5	1.1312	.0092
G3D6	1.1734	.0089
	1.1131	.0145
	1.1167	

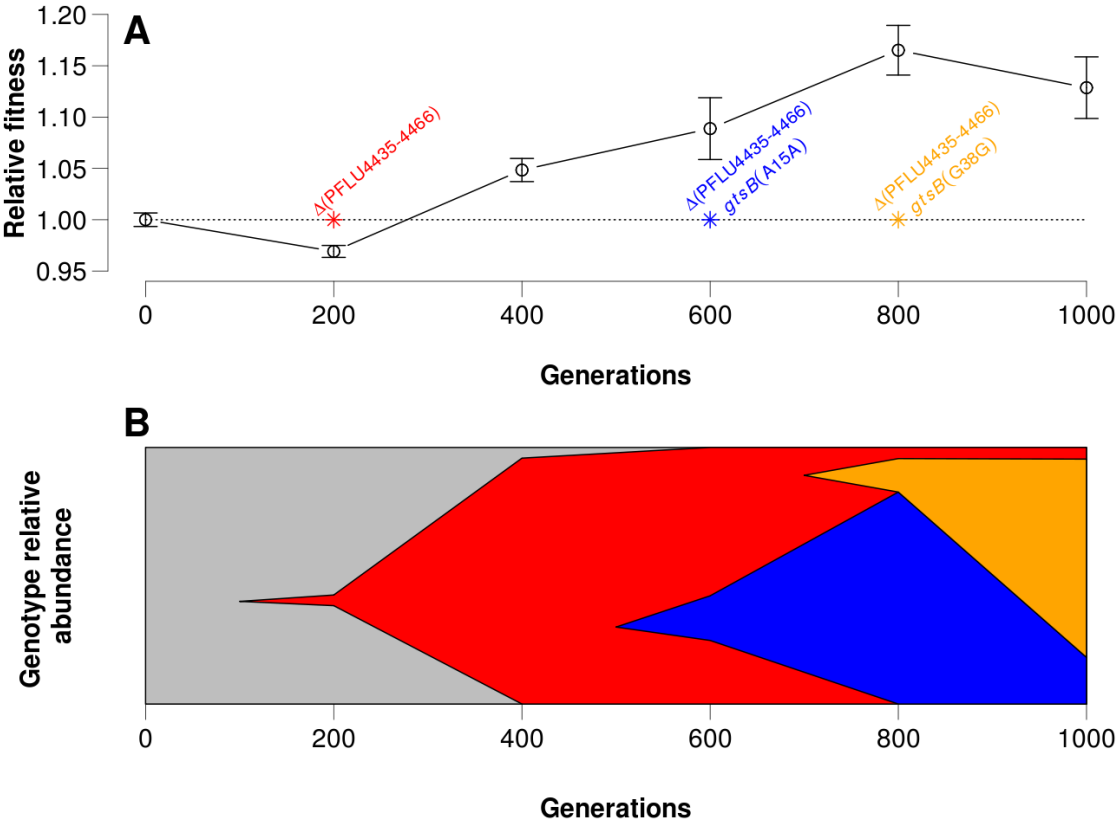


FIGURE 1: Dynamics of fitness increase and population composition change of a single *P. fluorescens* lineage in the course of adaptation to low glucose.

A) Population-wide fitness with respect to the wild-type (SBW25), mean±s.e.; N=3. * stands for the time of first detection.

B) Relative abundance of the wild-type ancestor (grey), Δ_{35kB} (red), Δ_{35kB}gtsB(A15A) (blue), Δ_{35kB}gtsB(G38G) (yellow) genotypes. The ensuing beneficial mutations dramatically swept through the evolving population, with near fixation of the deletion by generation 400, and high frequencies achieved by Δ_{35kB}+A15A and Δ_{35kB}+G38G genotypes. The wild type went under the detection threshold after the 600th generation. This is an example of periodic selection [153].

Reproduced from [122].

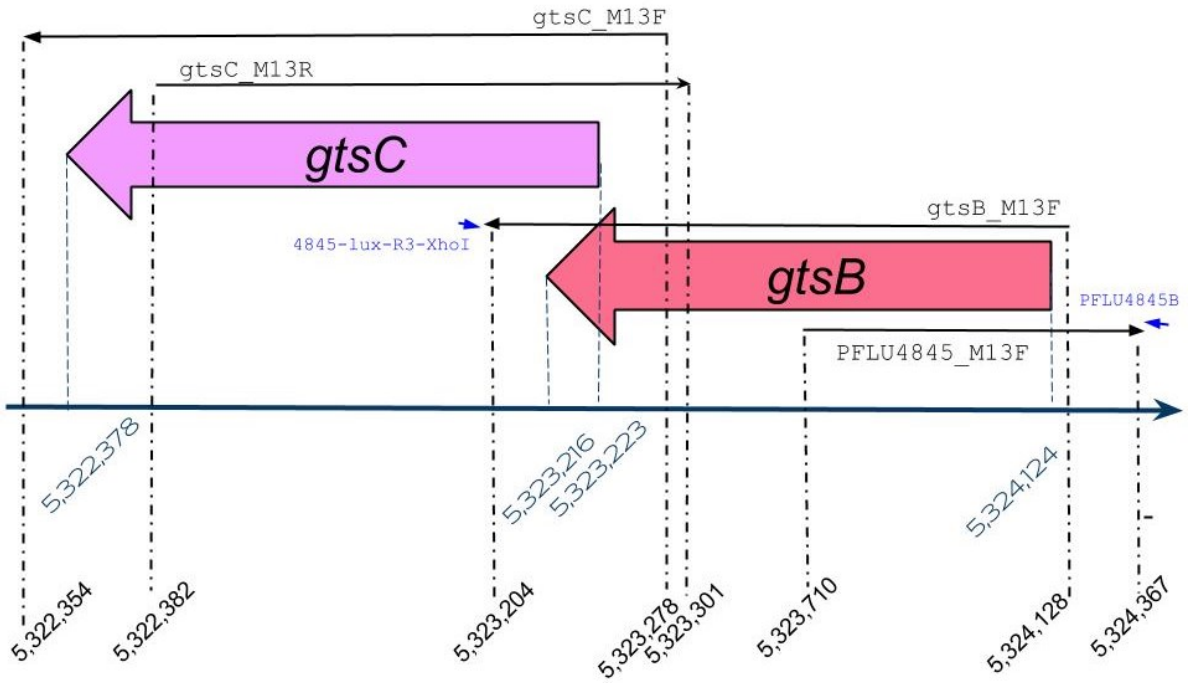


FIGURE 2: Sequencing coverage of *gtsB* (PFLU4845) and *gtsC* (PFLU4844) genic regions.

Black, sequencing primers with "universal" M13F or M13R tags. Blue, primers used to pair with sequencing primers to produce PCR products.

PCR primers:

PFLU4845B: GTGCGTTGTTTGATGTCGTG.

4845-lux-R3-XhoI: ACTGCGCTCGAGCTAGTCATTACGTTTGGTCCTC.

Sequencing primers:

gtsC_M13R: GGAAACAGCTATGACCATGCATTGCGAAGTTCAAGCGTA

gtsC_M13F: GTAAAACGACGGCCAGTCCAGTGCAATCCTGATGCT

gtsB_M13F: GTAAAACGACGGCCAGTTCCGACAGGCTGTAGTCCTT.

PFLU4845M13F: GTAAAACGACGGCCAGTGAGTCACGCAGGAGTTTGTCC.

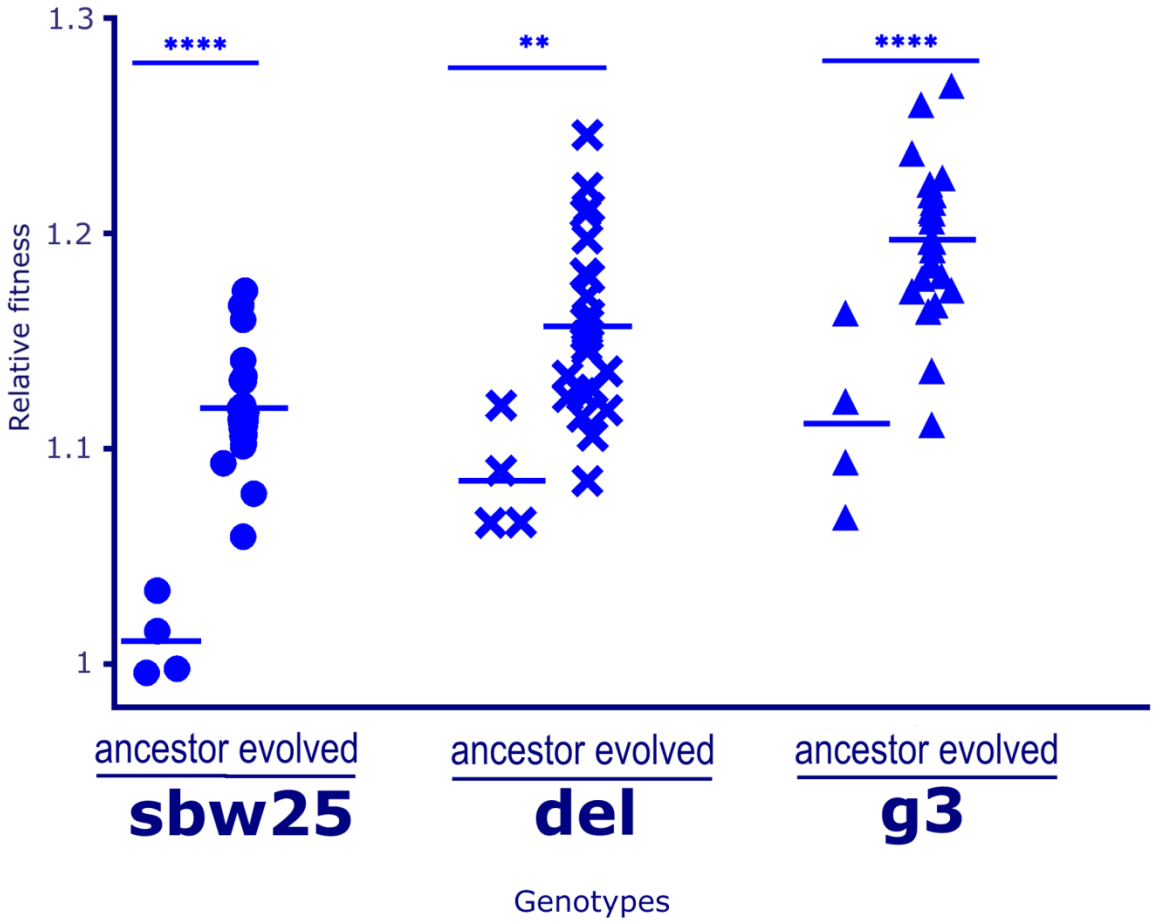


FIGURE 3: Fitness of each of SBW25, Δ_{35kB}(del), and G3 genotypes relative to the SBW25 wildtype, before and after adaptation. The leftmost column is the control of experimental SBW25 against SBW25 with a selectively neutral marker. The mean of 4 independent competitions did not deviate significantly from 1 ($t(3)=1.2198$, $p=.31$). Each data point represents a mean of N=4 replicate competitions for the evolved populations, and individual competitions for the ancestors. Horizontal bars are the means. The fitness increases after evolution were extremely statistically significant for SBW25 ($t(25)=7.86$, $p<.0001$), very statistically significant for Δ_{35kB} ($t(26)=3.47$, $p=.002$), and extremely statistically significant for G3 ($t(26)=4.38$, $p=.0002$).

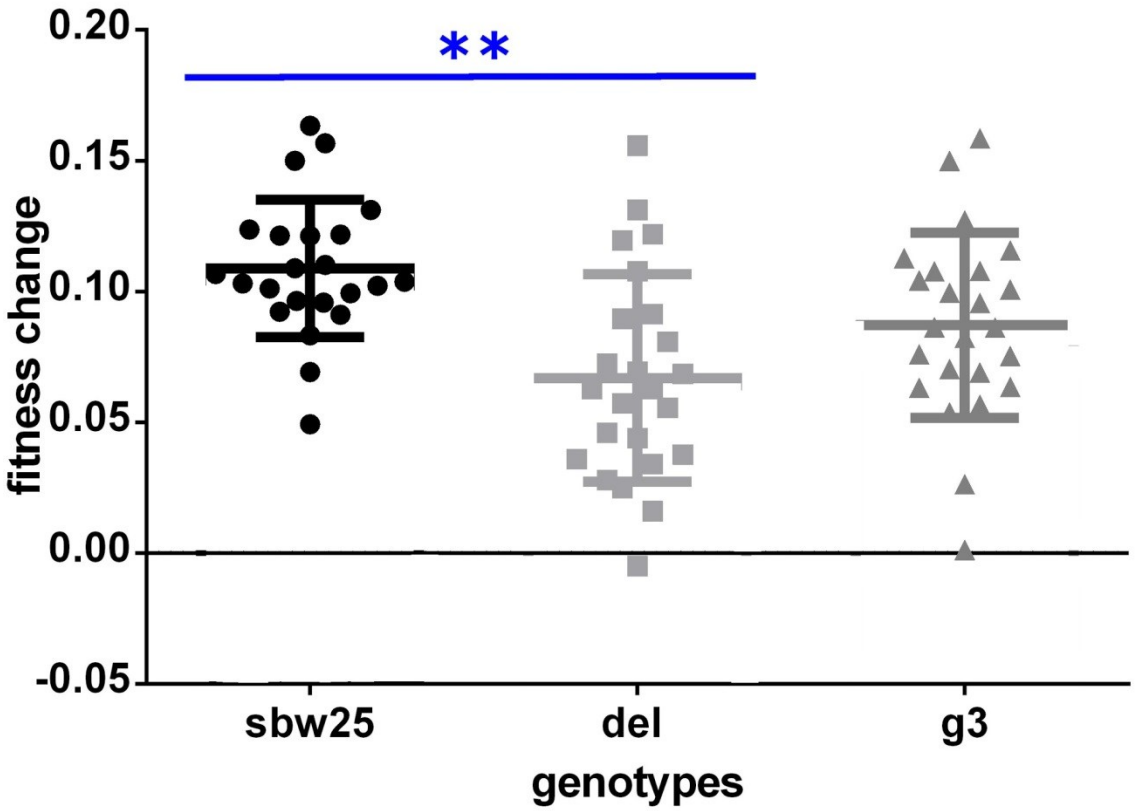


FIGURE 4: Relative adaptive fitness increase of each of the SBW25, Δ_{35kB}, and G3 genotypes. Each evolved data point was derived from a mean of N=4 replicate post-evolution competitions. The mean ±s.e. for each treatment is shown.

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment	0.02054	2	0.01027	F (2, 68) = 8.739	P = 0.0004
Residual	0.0799	68	0.001175		
Total	0.1004	70			
Tukey's test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value
sbw25 vs. del	0.04182	0.01785 to 0.06578	Yes	***	0.0002
sbw25 vs. g3	0.02167	-0.002293 to 0.04564	No	ns	0.0843
del vs. g3	-0.02014	-0.04385 to 0.003569	No	ns	0.1115

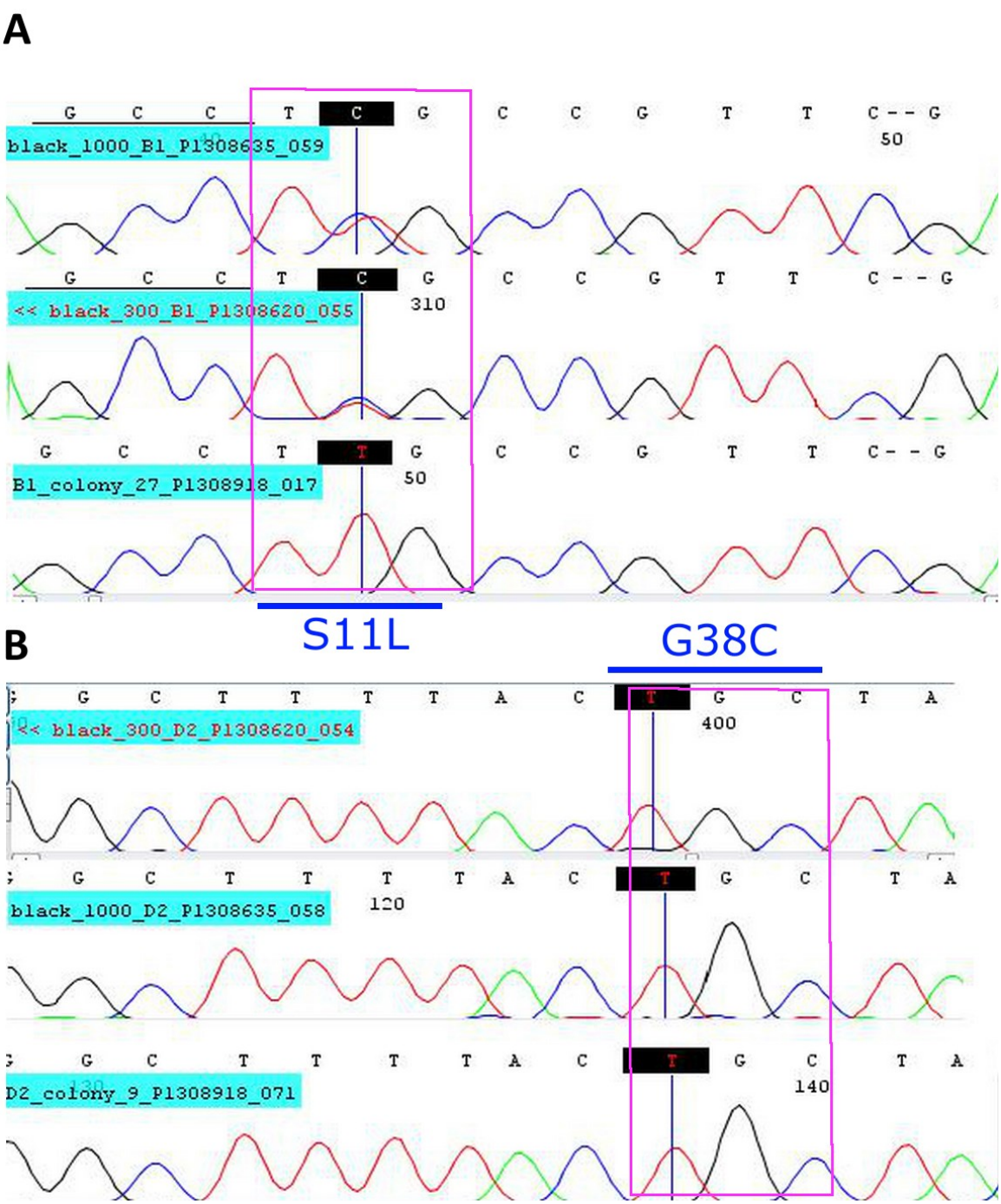


FIGURE 5: Representative sequencing chromatograms of *gtsB*.
(A): S11L {T**C**G>T**T**G} in Δ_{35kB} B1 population.
(B): G38C {**G**GC->**T**GC} in Δ_{35kB} D2 population.
In both panels, the top chromatograms show fragments resultant of population sequencing with PFLU4845_M13F primer, the middle chromatograms show fragments of population sequencing deriving from *gtsB*_M13F primer. In contrast, the bottom chromatograms in both panels show single colony PCR sequencing reads obtained with *gtsB*_M13F.

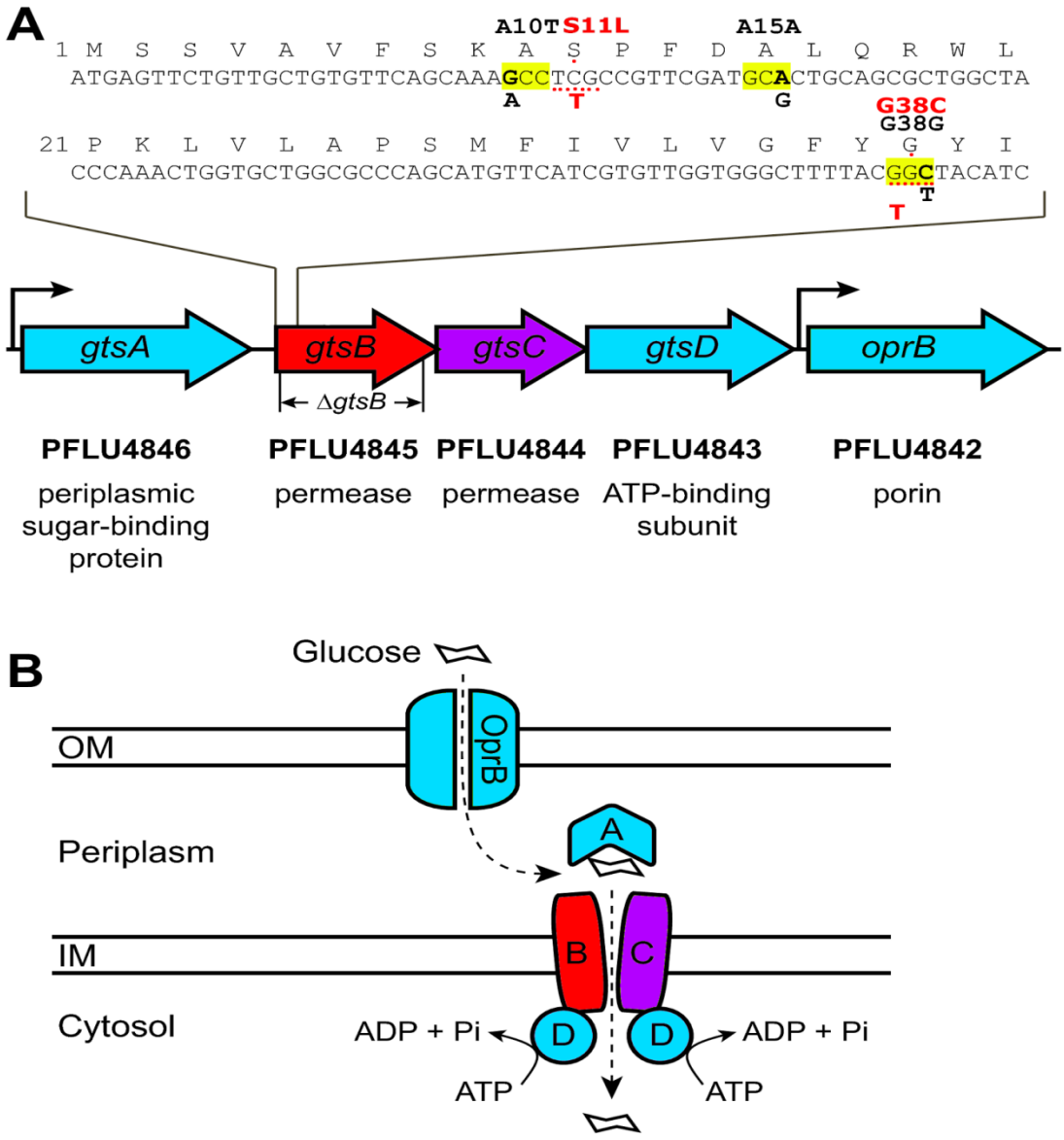
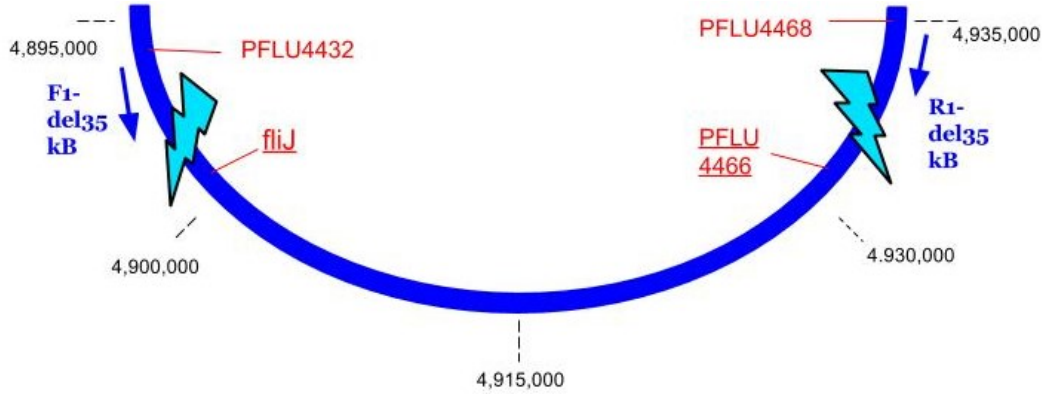


FIGURE 6: Genomic context and predicted functional organisation of the glucose transporter subunits, which the known naturally evolved synonymous (A15A and G38G) and nonsynonymous (A10T, S11L, G38C) mutations map to.
(A) The substitutions occurred in the 5' region of *gtsB*, but not *gtsC*, both of which encode permease subunits of the ABC transporter.
(B) Glucose passes through the outer membrane (OM) by facilitated diffusion through the OprB porin, is taken up by the periplasmic glucose-binding protein (GtsA), and transported across the ATP-dependent inner membrane (IM) channel.
Reproduced, with modifications, from [122].

A



B

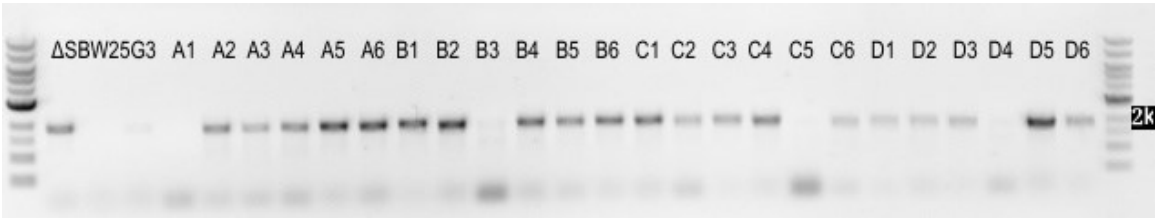


FIGURE 7: Δ_{35kB} deletion confirmation.

(A) A schematic of the region surrounding the 35kB deletion in Δ_{35kB} genotype. Primers encoding a 2 kB product in the presence of the deletion are shown as blue arrows. PCR primers used:

F1-del35kB ACTGCGGGATCC GGCATTGATACAACCGGAGT,

R1-del35kB ACTGCGACTAGT CCGATATTCGCCATGAATTG.

(B) Electrophoresis gel with the results of genotyping for Δ_{35kB} deletion. From left to right: $\Delta_{35kB,}$ SBW25, G3 controls, then Δ_{35kB} evolvants A1 to D6. '?' refers to a ?kb PCR product over the junction region created by the deletion and thus positively confirming it.

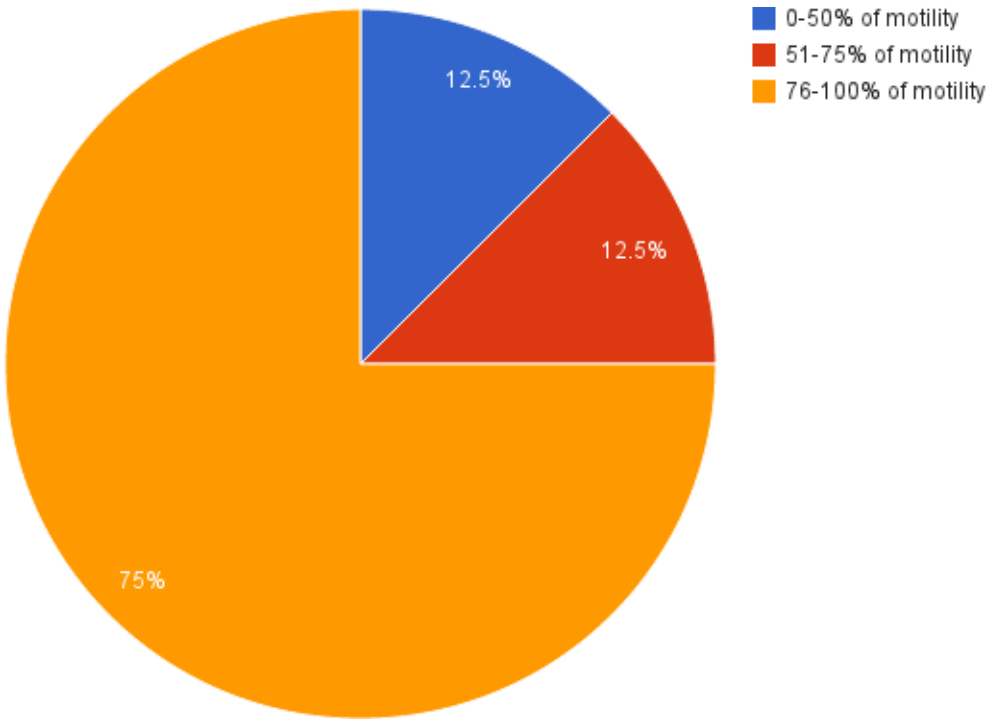


FIGURE 8: Semi-solid agar motility screen of the SBW25 evolvant populations, which displayed variability in the average motility. The frequency of phenotypic classes is proportionate to the areas of the circle. The isogenic SBW25 ancestor was 100% motile.

All of Δ_{35kB} and G3 evolvant populations tested non-motile, as did their respective isogenic ancestors.

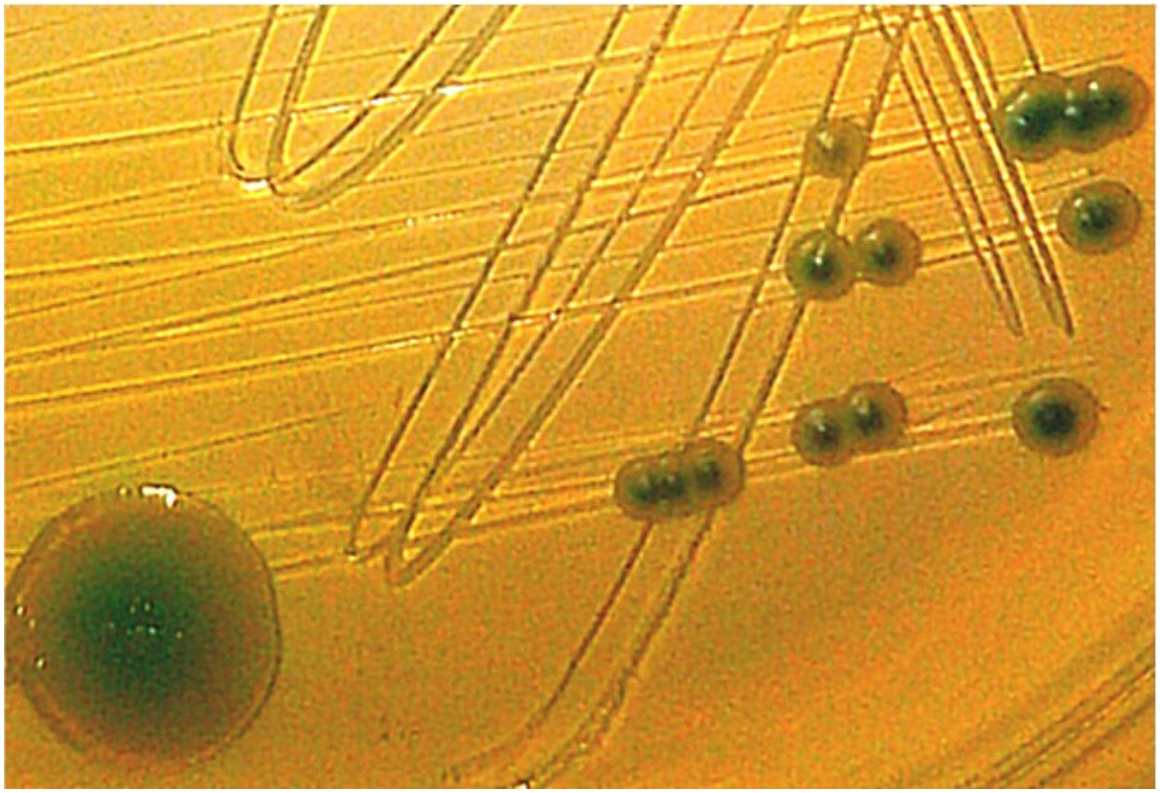


FIGURE 9: Slow-growing or non-culturable colonies detected in some replicates of SBW25, Δ_{35kB}, and G3 genotypes. Shown is the streak plating of a G3 population on a liquid broth plate. All colonies are of same age and have been exposed to the same conditions. *lacZ* marker expression (blue) suggests the absense of contamination. Left: a colony of regular size. Right: cryptic colonies.

Appendix A: Media recipes

Glucose x1/32 media (1L)

798mL autoclaved dH2O
200mL autoclaved 5x minimal salts
2mL MgSO4 1M solution
312μL autoclaved glucose 100x stock solution
100μL CaCl2 1M solution

5x minimal salts (1L)

33.9g Na2HPO4
15g KH2PO4
5g NH4Cl
2.5g NaCl
1L H2O

glucose 100x stock solution (200mL)

6.13g glucose
200mL H2O

competition X-gal agar

12g agar autoclaved in 800mL dH2O
200mL autoclaved 5x minimal salts
20mL 20% glucose solution
2mL MgSO4 1M solution
1mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)/DMF solution
100μL CaCl2 1M solution

Appendix B: Thermocycler programs

Initial denaturation	95°C	10 min
Cycling (x30)	95°C	30 s
	57°C	30 s
	72°C	1 min/kB
Final extension	72°C	5 min
Hold	4°C	∞

REFERENCES

1. Popper KR. Poverty of historicism. 1936.
2. Popper KR. Autobiography of Karl Popper. 1974.
3. Woese CR, Goldenfeld N. How the microbial world saved evolution from the Scylla of molecular biology and the Charybdis of the modern synthesis. Microbiol Mol Biol Reviews 2009 73(1): 14-21.
4. Ruse M. (ed) What the Philosophy of Biology is. 1989.
5. Dallinger WH. On the life-history of a minute septic organism: with an account of experiments made to determine its thermal death point. Proc. R. Soc. Lond. 1878 27: 332-350.
6. Bennett AF, Hughes BS. Microbial experimental evolution. Am J Physiol 2009 297(1): R17-R25.
7. Kassen R. Towards a general theory of adaptive radiation: insights from microbial experimental evolution. Annals N.Y. Academy of Sciences 2009 1168: 3-22.
8. Conrad TM, Lewis NE, Palssson BO. Microbial laboratory evolution in the era of genome-scale science. Mol Sys Biol 2011 7:509.
9. Drake JW, Charlesworth B, Charlesworth D, Crow JF. Rates of spontaneous mutation. Genetics 1998 148: 1667–1686.
10. Drake JW. The distribution of rates of spontaneous mutation over viruses, prokaryotes, and eukaryotes. Mol Strat Biol Evol 2006 870: 100-107.
11. Perfeito L, Fernades L, Mota C, Gordo I. Adaptive mutations in bacteria: high rate and small effects. Science 2007 317: 813-815.
12. Zietsch BP, Kuja-Halkola R, Walum H, Verweij KJN. Perfect genetic correlation between number of offspring and grandoffspring in an industrialized human population. Proc Natl Acad Sci U S A 2014 111(3): 1032-1036.
13. Dretske FI. Laws of Nature. Phil Sci 1977 44(2):248-268.
14. Bell G. Selection. 2008.
15. Dallinger WH. V. — The President's address. J Royal Micr Soc 1887
16. Darwin C. On the origin of species. 1859
17. Dobzhansky Th. Speciation as a stage in evolutionary divergence. Am Nat 1940 74 (753):312-321.
18. Schluter D. The ecology of adaptive radiation. 2000.
19. Simpson GG. The major features of evolution. 1953.
20. Rundle HD, Nagel JW, Boughman JW, Schluter D. Natural selection and parallel speciation in sympatric sticklebacks. Science 2001 287:306–308.
21. Berg L. Nomogenesis. M.I.T. Press 1969.
22. Schluter D, Clifford EA, Nemethy M, McKinnon JS. Parallel evolution and inheritance of quantitative traits. Am Nat 163(6):809-822.
23. Simpson GG. Principles of animal taxonomy. 1961.
24. Vavilov NI. The law of homologous series in variation. J Genet 1922 12:67–87.
25. Haldane JBS. The causes of evolution. 1932.

26. Wichman HA, Badgett MR, Scott LA, Boulianne, Bull JJ Different trajectories of
27. 1497-1507.J. Exceptional convergent evolution in a virus. Genet 1997 147(4) C.
28. Low AS, MacKenzie FM, Gould IM Booth IR. Protected environments allow parallel evolution of a bacterial pathogen in a patient subjected to long-term antibiotic therapy. Mol. Microbiol. 2001. 42: 619–630.
29. Riehle MM, Bennett AF, Long AD. Genetic architecture of thermal adaptation in Escherichia coli. Proc Natl Acad Sci U S A. 2001 98(2):525-30.
30. Anderson JB, Sirjusingh C, Parsons AB, Boone C, Wickens C, Cowen LE, Kohn LM. Mode of selection and experimental evolution of antifungal drug resistance in Saccharomyces cerevisiae. Genetics. 2003 163(4):1287-98.
31. Colosimo PF, Hosemann KE, Balabhadra S, Villarreal Jr G, Dickson M, Grimwood J, Schmutz J, Myers RM., Schluter D. and Kingsley DM Widespread parallel evolution in sticklebacks by repeated fixation of ectodysplasin alleles. Science 2005 307: 1928-1933.
32. Wood TE, Burke JM, Rieseberg LH. Parallel genotypic adaptation: when evolution repeats itself. Genetica 2005 123: 157–170.
33. Wolters G, Lennox JG, eds. Concepts, theories, and rationality in the biological sciences 1995.
34. Monod J. Chance and necessity. Vintage Books, New York. 1971
35. Pavé A. Necessity of chance: biological roulettes and biodiversity. Comp Rend Biol 2007. 330: 189–198.
36. Luria S, Delbrück, M. Mutations of bacteria from virus sensitivity to virus resistance. 1943. Genetics 28 (6): 491–511.
37. Brisson D. The directed mutation controversy in an evolutionary context. Crit Rev Microbiol 2003. 29 (1): 25–35.
38. Merlin F. Evolutionary chance mutation: a defense of the modern synthesis' consensus view. Philosophy & Theory in Biology 2010. 2:e103
39. Foster P. Stress-induced mutagenesis in bacteria. Crit Rev Biochem Mol Biol, 2007. 42: 373–397
40. Darwin, C. On the various contrivances by which British and foreign orchids are fertilised by insects, and on the good effects of intercrossing. 1862.
41. Ayala F. Darwin's explanation of design: from natural theology to natural selection. Infection, genetics and evolution 2009. 10: 839–842.
42. Gould S. Wonderful life: the Burgess Shale and the nature of history. Norton, New York. 1989.
43. Gould S. Eight little piggies: reflections in natural history. Norton, New York. 1993.
44. Popper K. The logic of scientific discovery. Rutledge, London 1959.
45. Kassen R. Towards a general theory of adaptive radiation: insights from microbial experimental evolution. Annals N.Y. Academy of Sciences 2009. 1168: 3-22.
46. Beatty J. Chance variation and evolutionary contingency: Darwin, Simpson, *The Simpsons*, and Gould." In Michael Ruse (ed.), Oxford Handbook of the Philosophy of Biology. Oxford University Press, Oxford. 2008.
47. Stern DL, Orgogozo V. Is genetic evolution predictable? Science 2009 323:746-751.
48. Otto SP. Two steps forward, one step back: the pleiotropic effects of favoured alleles. Proc. R. Soc. Lond. B 2004 271: 705-714.

49. Yukilevich R, Lachance J, Aoki F, True JR. Long-term adaptation of epistatic genetic networks. Evolution 2008 62(9): 2215-2235.
50. Garland T (Jr), Kelly SA. Phenotypic plasticity and experimental evolution. J Exp Biol 2006(209): 2344-2361.
51. Scoville AG, Phrender ME. Phenotypic plasticity facilitates recurrent rapid adaptation to introduced predators. Proc Natl Acad Sci U S A 2010 107(9): 4260-4263.
52. Pigliucci M. Evolution of phenotypic plasticity: where are we going now? Tren Ecol Evol 2005 20(9): 481-486.
53. DeWitt TJ, Sih A, Wilson DS. Costs and limits of phenotypic plasticity, Trend Ecol Evol 1998 13(2):77-81.
54. Toprak E, Veres A, Michel JB, Chait R, Hartl DL, Kishony R. Evolutionary paths to antibiotic resistance under dynamically sustained drug stress. Nat Genet. 2011 44(1): 101–105.
55. Blount ZD, Borland CZ, Lenski RE. Historical contingency and the evolution of a key innovation in an experimental population of *Escherichia coli* Proc Natl Acad Sci U S A 2008 105 (23) 7899-7906.
56. Lozovsky ER, Chookajorn T, Brown KM, Imwong M, Shaw PJ, Kamchonwongpaisan S, Neafsey DE, Weinreich DM, Hartl DL. Stepwise acquisition of pyrimethamine resistance in the malaria parasite. Proc Natl Acad Sci U S A 2009 106(29):12025-30.
57. Crandall KA, Kelsey CR, Imamichi H, Lane HC, Salzman NP. Parallel evolution of drug resistance in HIV: failure of nonsynonymous/synonymous substitution rate ratio to detect selection. Mol Biol Evol. 1999 16(3):372-82.
58. Lenski RE, Travisano M. Dynamics of adaptation and diversification: a 10,000-generation experiment with bacterial populations. Proc Natl Acad Sci U S A 1994 91(15):6808-14.
59. de Visser JAGM, Hermisson J, Wagner GP, Ancel Meyers L, Bagheri-Chaichian H, Blanchard JL, Chao L, Cheverud JM, Elena SF, Fontana W, Gibson G, Hansen TF, Krakauer D, Lewontin RC, Ofria C, Rice SH, von Dassow G, Wagner A, Whitlock MC. Perspective: Evolution and detection of genetic robustness. Evolution. 2003 57(9):1959-72.
60. Swallow JG, Garland T Jr. Selection experiments as a tool in evolutionary and comparative physiology: insights into complex traits--an introduction to the Symposium. Integr Comp Biol. 2005 ;45(3):387-90.
61. Keuth H. The philosophy of Karl Popper. 2004.
62. Beatty J. Replaying life's tape. J Phil. 2006 103(7):336-362.
63. Arnold M.L. Evolution through genetic exchange. 2006.
64. Keeling PJ, Palmer JD. Horizontal gene transfer in eukaryotic evolution. Nat Rev Genet 2008(9):605-618.
65. Dunning Hotopp JC, Clark ME, Oliveira DC, Foster JM, Fischer P, Muñoz Torres MC, Giebel JD, Kumar N, Ishmael N, Wang S, Ingram J, Nene RV, Shepard J, Tomkins J, Richards S, Spiro DJ, Ghedin E, Slatko BE, Tettelin H, Werren JH. Widespread lateral gene transfer from intracellular bacteria to multicellular eukaryotes. Science 2007 317(5845):1753-1756.
66. Pace JK 2nd, Gilbert C, Clark MS, Feschotte C. Repeated horizontal transfer of a DNA transposon in mammals and other tetrapods. Proc Natl Acad Sci U S A 2008 105(44):17023-8.

67. Popper K. The poverty of historicism. 1957.
68. Zhang G, Cowled C, Shi Z, Huang Z, Bishop-Lilly KA, Fang X, Wynne JW, Xiong Z, Baker ML, Zhao W, Tachedjian M, Zhu Y, Zhou P, Jiang X, Ng J, Yang L, Wu L, Xiao J, Feng Y, Chen Y, Sun X, Zhang Y, Marsh GA, Crameri G, Broder CC, Frey KG, Wang LF, Wang J. Comparative analysis of bat genomes provides insight into the evolution of flight and immunity. Science. 2013 339(6118):456-60.
69. Garland T Jr., Rose MR (eds). Experimental evolution: concepts, methods, and applications of selection experiments. 2009.
70. Quinn JF, Dunham AE. On hypothesis testing in ecology and evolution. 1983 Am Nat 122(5):602-617.
71. Losos JB, Jackman TR, Larson A, Queiroz K, Rodriguez-Schettino L. Contingency and determinism in replicated adaptive radiations of island lizards. Science. 1998 279(5359):2115-8.
72. Burbrink FT, Chen X, Myers EA, Brandley MC, Pyron RA. Evidence for determinism in species diversification and contingency in phenotypic evolution during adaptive radiation. Proc Biol Sci. 2012 279(1748):4817-26.
73. Gamble T, Greenbaum E, Jackman TR, Russell AP, Bauer AM. Repeated origin and loss of adhesive toepads in geckos. PLoS One. 2012;7(6):e39429.
74. Kidd MR, Duftner N, Koblmüller S, Sturmbauer C, Hofmann HA. Repeated parallel evolution of parental care strategies within Xenotilapia, a genus of cichlid fishes from Lake Tanganyika. PLoS One. 2012;7(2):e31236.
75. Bedhomme S, Lafforgue G, Elena SF. Genotypic but not phenotypic historical contingency revealed by viral experimental evolution. BMC Evol Biol. 2013 13:46.
76. Eroukhmanoff F, Hargeby A, Arnberg NN, Hellgren O, Bensch S, Svensson EI. Parallelism and historical contingency during rapid ecotype divergence in an isopod. J Evol Biol 2009, 22:1098-1110.
77. Travisano M, Mongold JA, Bennett AF, Lenski RE. Experimental tests of the roles of adaptation, chance, and history in evolution. Science. 1995 267(5194):87-90.
78. Travisano M, Vasi F, Lenski RE. Long-term experimental evolution in Escherichia coli. III. Variation among replicate populations in correlated responses to novel environments. Evolution 1995 49:189-200.
79. Saxer G, Doebeli M, Travisano M. The repeatability of adaptive radiation during long-term experimental evolution of Escherichia coli in a multiple nutrient environment. PLoS One. 2010 Dec 2;5(12):e14184.
80. Cooper TF, Rozen DE, Lenski RE. Parallel changes in gene expression after 20,000 generations of evolution in Escherichia coli. Proc Natl Acad Sci USA 2003 100: 1072–1077.
81. Fong SS, Joyce AR, Palsson BO Parallel adaptive evolution cultures of Escherichia coli lead to convergent growth phenotypes with different gene expression states. Genome Res 2005 15: 1365–1372.
82. Stoebel DM, Hokamp K, Last MS, Dorman CJ Compensatory evolution of gene regulation in response to stress by Escherichia coli lacking RpoS. PLoS Genet 2009 5: e1000671.
83. Orr HA. The genetic theory of adaptation: a brief history. Nat Reviews 2005 6:119-127.

84. Conrad T.M, Lewis NE, Palsson BO. Microbial laboratory evolution in the era of genome-scale science. Mol Sys Biol 2011 7:509.
85. Fisher RA. The genetical theory of natural selection. 1930.
86. Kassen R, Bataillon T. Distribution of fitness effects among beneficial mutations before selection in experimental populations of bacteria. Nature Genet 2006 38:484 - 488.
87. Bulmer MG. The mathematical theory of quantitative genetics. 1980.
88. Orr HA. The Population Genetics of Adaptation: The distribution of factors fixed during adaptive evolution. Evolution 1998 52(4): 935-949.
89. Perfeito L, Fernandez L, Mota C, Gordo I. The distribution of fitness effects of new mutations. Science 2007 317:813-815.
90. Wiser MJ, Ribeck N, Lenski RE. Long-term dynamics of adaptation in asexual populations. Science. 2013 342(6164):1364-7.
91. Frenkel EM, Good BH, Desai MM. The fates of mutant lineages and the distribution of fitness effects of beneficial mutations in laboratory budding yeast populations. Genet 2014 doi: 10.1534/genetics.113.160069.
92. Rozen DE, de Visser JAGM, Gerrish PJ. Fitness effects of fixed beneficial mutations in microbial populations, Curr Biol, 2002 12(12):1040-1045.
93. Eyre-Walker A, Keightley PD. The distribution of fitness effects of new mutations. Nature Reviews Genet 2007 8: 610-618.
94. Hardin G. The tragedy of the commons. Science 1968 162(3859):1243-8.
95. MacLean RC, Perron GG, Gardner A. Diminishing returns from beneficial mutations and pervasive epistasis shape the fitness landscape for rifampicin resistance in Pseudomonas aeruginosa. Genet 2010 186: 1345–1354.
96. Chevin L.-M, Martin G, Lenormand T. Fisher's model and the genomics of adaptation: Restricted pleiotropy, heterogenous mutation, and parallel evolution. Evolution 2010 64(11):3213-3231.
97. Turner JRG. Fisher's evolutionary faith and the challenge of mimicry. Ox Surv Evol Biol 1985 2:159-196.
98. Gould SJ, Lewontin RC. The spandrels of San Marco and the Panglossian paradigm: a critique of the adaptationist programme. Proc R Soc Lond B Biol Sci. 1979 205(1161):581-98.
99. Orr HA. The population genetics of beneficial mutations. Phil. Trans. R. Soc. B 2010 365: 1195–1201.
100. Dobzhansky T. How do the genetic loads affect the fitness of their carriers in Drosophila populations? Am Nat 1964 98(900):151-166.
101. Dobzhansky T, Spassky B. Genetics of Natural Populations. Xl. Heterotic and deleterious effects of recessive lethals in populations of Drosophila pseudoobscura. Genetics. 1968 59(3): 411–425.
102. Dobzhansky T, Pavlovsky O, Spassky B, Spassky N. Genetics of Natural Populations. Xxiii. Biological role of deleterious recessives in populations of Drosophila pseudoobscura. Genetics. 1955 November; 40(6): 781–796.
103. Dobzhansky T, Spassky B. Genetics of natural populations. Xxxiv. Adaptive norm, genetic load and genetic elite in Drosophila pseudoobscura. Genetics. 1963 48(11): 1467–1485.
104. Orr HA. The distribution of fitness effects among beneficial mutations. Genetics 2003 163: 1519–1526.
105. Kimura M. The neutral theory of molecular evolution. 1983.

106. Kimura M. The neutral theory of molecular evolution: A review of recent evidence. Jpn J Genet 1991 66: 367-386.
107. Ohta T. The nearly neutral theory of molecular evolution. Ann Rev Ecol System1992 23:263-286.
108. Kimura M. Preponderance of synonymous changes as evidence for the neutral theory of molecular evolution. Nature 1977 267,:275-276.
109. Fay JC, Wyckoff GJ, Wu CI. Testing the neutral theory of molecular evolution with genomic data from Drosophila. Nature 2001 415: 1024-1026.
110. Kreitman M. The neutral theory is dead. Long live the neutral theory. BioEssays 1996 18(8): 678–683.
111. Lind PA, Berg OG, Dandersson DI. Mutational robustness of ribosomal protein genes. Science 2010 330:825-827.
112. Sauna ZE, Kimchi-Sarfaty C. Understanding the contribution of synonymous mutations to human disease. Nat Rev Genet. 2011 12(10):683-91.
113. Parmley JL, Hurst LD. How do synonymous mutations affect fitness? Bioessays. 2007 29(6):515-9.
114. Kimchi-Sarfaty C, Oh JM, Kim IW, Sauna ZE, Calcagno AM, Ambudkar SV, Gottesman MM. A "silent" polymorphism in the MDR1 gene changes substrate specificity. Science. 2007 26;315(5811).
115. Zhang G, Hubalewska M, Ignatova Z. Transient ribosomal attenuation coordinates protein synthesis and co-translational folding. Nat Struct Mol Biol. 16(3):274-80.
116. Shabalina SA, Spiridonov NA, Kashina A. Sounds of silence: synonymous nucleotides as a key to biological regulation and complexity. Nucleic Acids Res. 2013 41(4):2073-94.
117. Chamary JV, Parmley JL, Hurst LD. Hearing silence: non-neutral evolution at synonymous sites in mammals. Nat Rev Genet. 2006 (2):98-108.
118. JV Chamary, Hurst LD. Evidence for selection on synonymous mutations affecting stability of mRNA secondary structure in mammals. Genome Biol. 2005; 6(9): R75.
119. Parmley JL, Chamary JV, Hurst LD. Evidence for purifying selection against synonymous mutations in mammalian exonic splicing enhancers. Mol Biol Evol. 2006 23(2):301-9.
120. Plotkin JB, Kudla G. Synonymous but not the same: the causes and consequences of codon bias. Nat Rev Genet. 2011 Jan;12(1):32-42.
121. Sharp PM, Bailes E, Grocock RJ, Peden JF, Sockett RE. Variation in the strength of selected codon usage bias among bacteria. Nucleic Acids Res. 33(4):1141-53.
122. Bailey SF, Hinz A, Kassen R. Adaptive synonymous mutations in an experimentally evolved population. 2014 *(accepted at Nature Communications)*.
123. Barrick JE, Lenski RE. Genome dynamics during experimental evolution. Nat Rev Genet 2013 14:827-839.
124. Cuevas JM, Domingo-Calap P, Sanjuán R. The fitness effects of synonymous mutations in DNA and RNA viruses. Mol Biol Evol. 2012 29(1):17-20.
125. Schenk MF, Szendro IG, Krug J, de Visser JAGM. Quantifying the adaptive potential of an antibiotic resistance enzyme. PLoS Genet. 2012 8(6): e1002783.

126. Agashe D, Martinez-Gomez NC, Drummond DA, Marx CJ. Good codons, bad transcript: large reductions in gene expression and fitness arising from synonymous mutations in a key enzyme. Mol Biol Evol. 2013 30(3):549-60.
127. del Castillo T, Ramos J, Rodríguez-Herva J, Fuhrer T, Sauer U, Duque E. Convergent peripheral pathways catalyze initial glucose catabolism in *Pseudomonas putida*: Genomic and Flux Analysis. Journal of Bacteriology 2007 189: 5142-5152.
128. Khan AI, Dinh DM, Schneider D, Lenski RE, Cooper TF. Negative epistasis between beneficial mutations in an evolving bacterial population. Science. 332(6034):1193-6.
129. Chou HH, Chiu HC, Delaney NF, Segrè D, Marx CJ. Diminishing returns epistasis among beneficial mutations decelerates adaptation. Science. 2011 332(6034):1190-2.
130. Kvitek DJ, Sherlock G. Reciprocal sign epistasis between frequently experimentally evolved adaptive mutations causes a rugged fitness landscape. PLoS Genet. 7(4):e1002056.
131. Hancock REW, Carey AM. Protein D1 — A glucose-inducible, pore-forming protein from the outer membrane of Pseudomonas aeruginosa. FEMS Microbiol Let. 1980 8(2): 105-109.
132. Wylie JL, Worobec EA. Substrate specificity of the high-affinity glucose transport system of Pseudomonas aeruginosa. Can J Microbiol. 1993 39(7):722-5.
133. Wylie JL, Bernegger-Egli C, O'Neil JD, Worobec EA. Biophysical characterization of OprB, a glucose-inducible porin of Pseudomonas aeruginosa. J Bioenerg Biomembr. 1993 (5):547-56.
134. Wylie JL, Worobec EA. The OprB porin plays a central role in carbohydrate uptake in Pseudomonas aeruginosa. J Bacteriol. 1995 177(11):3021-6.
135. Gould SJ The structure of evolutionary theory. 2002.
136. Spiers AJ, Buckling A, Rainey PB. The causes of Pseudomonas diversity. Microbiol 2000 146 (10):2345-50.
137. Bailey MJ, Lilley AK, Thompson IP, Rainey PB, Ellis RJ. Site directed chromosomal marking of a fluorescent pseudomonad isolated from the phytosphere of sugar beet; stability and potential for marker gene transfer. Mol Ecol. 1995 4(6):755-63.
138. Winsor GL, Lam DK, Fleming L, Lo R, Whiteside MD, Yu NY, Hancock RE, Brinkman FS. Pseudomonas Genome Database: improved comparative analysis and population genomics capability for Pseudomonas genomes. Nucleic Acids Res. 2011 39(Database issue):D596-600.
139. Silby MW, Cerdeño-Tárraga AM, Vernikos GS, Giddens SR, Jackson RW, Preston GM, Zhang XX, Moon CD, Gehrig SM, Godfrey SA, Knight CG, Malone JG, Robinson Z, Spiers AJ, Harris S, Challis GL, Yaxley AM, Harris D, Seeger K, Murphy L, Rutter S, Squares R, Quail MA, Saunders E, Mavromatis K, Brettin TS, Bentley SD, Hothersall J, Stephens E, Thomas CM, Parkhill J, Levy SB, Rainey PB, Thomson NR. Genomic and genetic analyses of diversity and plant interactions of Pseudomonas fluorescens. Genome Biol. 2009;10(5):R51.
140. Travisano M, Lenski RE. Long-term experimental evolution in Escherichia coli. IV. Targets of selection and the specificity of adaptation. Genetics. 1996 143(1):15-26.

141. Melnyk AH, Kassen R. Adaptive landscapes in evolving populations of Pseudomonas fluorescens. Evolution. 65(11):3048-59.
142. van Overbeek LS, Eberl L, Givskov M, Molin S, van Elsas JD. Survival of, and induced stress resistance in, carbon-starved Pseudomonas fluorescens cells residing in soil. Appl Environ Microbiol. 1995 61(12):4202-8.
143. Reinhard F, Miyazaki R, Pradervand N, van der Meer JR. Cell differentiation to "mating bodies" induced by an integrating and conjugative element in free-living bacteria. Curr Biol. 2013 23(3):255-9.
144. Davis BM, Waldor MK. Horizontal gene transfer: linking sex and cell fate. Curr Biol. 2013 23(3):R118-9.
145. Silby MW, Cerdeño-Tárraga AM, Vernikos GS, Giddens SR, Jackson RW, Preston GM, Zhang XX, Moon CD, Gehrig SM, Godfrey SA, Knight CG, Malone JG, Robinson Z, Spiers AJ, Harris S, Challis GL, Yaxley AM, Harris D, Seeger K, Murphy L, Rutter S, Squares R, Quail MA, Saunders E, Mavromatis K, Brettin TS, Bentley SD, Hothersall J, Stephens E, Thomas CM, Parkhill J, Levy SB, Rainey PB, Thomson NR. Genomic and genetic analyses of diversity and plant interactions of Pseudomonas fluorescens. Genome Biol. 2009;10(5):R51.
146. Andreani NA, Martino ME, Fasolato L, Carraro L, Montemurro F, Mioni R, Bordin P, Cardazzo B. Tracking the blue: A MLST approach to characterise the Pseudomonas fluorescens group. Food Microbiol 2014 (39): 116-126.
147. Sarkar SF, Guttman DS. Evolution of the core genome of Pseudomonas syringae, a highly clonal, endemic plant pathogen. Appl Environ Microbiol. 2004 70(4): 1999–2012.
148. Treves DS, Manning S, Adams J. Repeated evolution of an acetate-crossfeeding polymorphism in long-term populations of Escherichia coli. Mol. Biol. Evol. 1998 15:789–797.
149. Rozen DE, Lenski RE. Long-term experimental evolution in Escherichia coli VIII. Dynamics of a balanced polymorphism. Am. Nat. 2000 155:24–35.
150. Turcotte MM, Corrin MSC, Johnson MTJ. Adaptive evolution in ecological communities. PLoS Biol 2012 10(5): e1001332.
151. Rojo F. Carbon catabolite repression in Pseudomonas: optimizing metabolic versatility and interactions with the environment. FEMS Microbiol Rev. 2010 34(5):658-84.
152. McElroy KE, Hui JG, Woo JK, Luk AW, Webb JS, Kjelleberg S, Rice SA, Thomas T. Strain-specific parallel evolution drives short-term diversification during Pseudomonas aeruginosa biofilm formation. Proc Natl Acad Sci U S A. 2014 111(14):E1419-27.
153. Atwood KS, Schneider LK, Ryan FJ. Periodic selection is Escherichia coli. Proc Natl Acad Sci U S A. 37: 146-155.