12-2014

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Recommended Citation

Julian Adams, Frank Rosenzweig, Experimental microbial evolution: history and conceptual underpinnings, Genomics, Volume 104, Issue 6, Part A, 2014, Pages 393-398, https://doi.org/10.1016/j.ygeno.2014.10.004

Genomics 104 (2014) 393–398

Contents lists available at ScienceDirect

Genomics

journal homepage: www.elsevier.com/locate/ygeno

Review

Experimental microbial evolution: history and conceptual underpinnings

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ARTICLE INFO

Article history:
Received 2 October 2014
Accepted 6 October 2014
Available online 12 October 2014

Keywords:
Continuous culture
Chemostat
Serial dilution
DNA sequencing
Bioinformatics
Fitness
Experimental microbial evolution

ABSTRACT

We chronicle and dissect the history of the field of Experimental Microbial Evolution, beginning with work by Monod. Early research was largely carried out by microbiologists and biochemists, who used experimental evolutionary change as a tool to understand structure–function relationships. These studies attracted the interest of evolutionary biologists who recognized the power of the approach to address issues such as the tempo of adaptive change, the costs and benefits of sex, parallelism, and the role which contingency plays in the evolutionary process. In the 1980s and 1990s, an ever-expanding body of microbial, physiological and biochemical data, together with new technologies for manipulating microbial genomes, allowed such questions to be addressed in ever-increasing detail. Since then, technological advances leading to low-cost, high-throughput DNA sequencing have made it possible for these and other fundamental questions in evolutionary biology to be addressed at the molecular level.

Contents

1. Introduction

In its short history the field of experimental microbial evolution (EME) has brought together biologists from disciplines in Biology that traditionally had little in common; the so-called "skin-in" sciences of molecular biology and microbiology on the one hand and the "skin-out" science of evolutionary biology on the other. In many universities, practitioners were located in different departments, and even in different schools of colleges, further limiting their contact. The "glue" underlying the emergence of this new hybrid field has been the use of a common set of techniques and experimental approaches, perhaps the most significant of which has been the use of the chemostat. Interactions between practitioners in these disparate fields have often been creative, resulting in significant new advances. Yet often the philosophical motivations for research in EME illustrate the same traditional dichotomy that exists between "skin-in" and "skin-out" biology; the elucidation of structure–function relationships of intracellular molecular components on the one hand, and the analysis and documentation of emergent population phenomena on the other. In the following sections we expand on the issues mentioned above.

2. EME: early beginnings

Although a few reports appeared in the early part of the twentieth century which documented changes in microbial populations, the first concrete steps in the creation of the new field of EME were made by Jacques Monod in the 1940s when he described the construction,

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http://dx.doi.org/10.1016/j.ygeno.2014.10.004
0888-7543/

operation and kinetics of growth in a continuously growing culture where growth was limited by the concentration of a single nutrient [1]. He called the device a "Bactogen" — the term "chemostat" only came into usage in the 1950s when it was coined by Novick and Szilard [2]. The motivation for his work with the "Bactogen" had little to do with evolution: rather, Monod was interested in growth rates under limiting nutrient concentration and the phenomenon of diauxic growth. Nevertheless, Monod clearly realized that genetic, that is, evolutionary changes would occur in the continuous cultures and that these would have intrinsic interest when he wrote [3] that,

> "*[L]a technique de culture continue trouverait sans doute des applications intéressantes dans l'analyse de mutabilité…on ne peut négliger a priori les facteurs de sélection*".("*Without doubt, the technique of continuous culture will find applications in the analysis of mutability…a priori we cannot disregard factors of selection*".)

Monod indicated that he would enlarge on this issue in a future communication with co-workers, Torriani and Doudoroff — a paper that apparently was never published.

The beginning of the 1950s saw the publication of three papers that are frequently cited as the first papers in EME to describe and analyze genetic changes in continuous cultures of microorganisms. Each employed a different culture technique, Novick and Szilard [2] used the chemostat, Bryson and Szybalski [4] used the turbidostat, while Atwood, Schneider and Ryan [5] used serial dilution. Though the culture techniques were different, the themes of these papers were the same: namely the occurrence of mutants that were selectively favored during extended growth. These papers could therefore be considered in a general sense microbiological equivalents of work that was appearing frequently in the experimental population genetics literature during that era, work that described and measured selective differences between mutants in the laboratory using the population geneticists' favorite genus of the time — *Drosophila*.

3. Next steps

Much of the work in EME over the next twenty or more years dealt with the analysis of mutants that were selectively favored during extended growth, and so can be considered to be in the same genre as the papers of Novick and Szilard [2], Bryson and Szybalski [4], and Atwood, Schneider and Ryan [5]. The collected papers in a monograph on Evolutionary Biology published in 1984 [6] reflected the trend of the era — an almost exclusive focus on evolutionary change in enzyme activity on poor substrates. For example, Hartley and his group reported on the evolutionary changes in the structure and regulation of ribitol dehydrogenase activity of *Klebsiella aerogenes* growing on xylitol, a poor substrate in chemostat culture [7], while Clarke and her group described changes in the structure and regulation of amidase in *Pseudomonas aeruginosa* growing in plate culture on novel substrates [8]. An interesting variant on this theme is illustrated by the work by Hall on the evolution of *Escherichia coli* possessing a deletion of the *lacZ* gene coding for β-galactosidase growing on lactose as the sole carbon source [9]. Repeated streaking on Petri plates revealed the existence of a second enzyme capable of hydrolyzing lactose, termed by Hall as EBG ("Evolved Beta-Galactosidase"). It can be argued that these workers, for the most part, microbiologists and biochemists, were using evolution merely as a tool to gain insights into enzyme structure and the genetic structure of the organism. While the primary goal of these studies was an increased understanding of the biochemistry and physiology of the microbial species studied, they clearly had broader significance, increasing our knowledge of the biology of a wide range of organisms, both unicellular and multicellular, and advancing understanding of the genetic basis of evolutionary change. Thus, the observations that regulatory — that is constitutive mutations, and gene duplications often precede selection for any structural changes in enzymes [*e.g.*, 7], provided the first concrete evidence for the adaptive significance of regulatory changes in evolution — a finding that has become a cornerstone of our understanding of the genetics of speciation. Moreover, these early observations that gene duplication was an important mechanism for genetic adaptation foreshadowed later work that uncovered large scale genomic changes in both prokaryotes and eukaryotes [10–12]. Similarly, the understanding that genomes may possess inactive or redundant elements was confirmed and vastly expanded on, by genomic DNA sequence analysis many years later.

A variation on this theme was stimulated by a consideration of cell energetics. Conservation of energy by the elimination of unnecessary cellular components would, it was argued, be adaptively favored. Early experiments by Spiegelman and his group showing that the repeated passaging of the virus Qβ, selecting for shorter passage times, resulted in a virus with a genome only 15% the size of the original virus [13–15], foreshadowed much later work [*cf.* 16]. In addition, experiments by Zamenhof and Eichhorn [17] suggested that histidine and tryptophan auxotrophs of *Bacillus subtilis* were strongly selectively favored over the corresponding prototroph, when the appropriate amino acid histidine or tryptophan was provided in the growth medium. The sizes of the selective advantage (estimated selective coefficients are 0.33–0.47), however, were inconsistent with strict energetic considerations, and suggested instead that the prototrophic and auxotrophic strain pairs harbored other differences other than those in amino acid biosynthetic operons. Indeed, a careful study by Dykhuizen [18] comparing different tryptophan auxotrophs with the otherwise isogenic tryptophan prototroph failed to show consistent advantages based on strict energetic considerations. Only the inverse experiment, measuring the selective disadvantage of protein *over*production of a protein — by Lac constitutive strains gave results roughly consistent with energetic considerations [19].

4. Evolutionary biologists recognize the power of the experimental system

The active involvement of evolutionary biologists, recognizing the power of a microbial system to address evolutionary questions, resulted in a different set of questions being addressed. To this group of scientists, the advantages of microbial systems were principally three-fold, i) the ability to strictly regulate physical aspects of the environment, ii) the short generation times, and iii) "living fossil records" of evolutionary changes which could be maintained by cryopreservation. (These advantages were particularly exploited by Lenski and coworkers in their work, [*e.g.*, 20]). For the first time then, questions such as the evolution of predator–prey relationships could be addressed experimentally. These studies which took advantage of host-parasite interactions such as those between bacteria and bacteriophages [21–24], allowed the analysis of the dynamics of the occurrence of sequential resistance and pathogenicity mutations in a detailed way that hitherto had not been possible in earlier studies with flax and flax rust [25,26]. The aforementioned studies provided considerable insight into the natural history of microbial populations. However, for some investigators, microorganisms were merely biological simulators of processes occurring in other (more complex and multicellular) organisms, and often did not exploit the growing body of genetic, physiological and biochemical information available for species such as *E. coli* and *Saccharomyces cerevisiae*. In contrast to the work described in the previous section, the results may have had limited or no relevance to organism of choice; the system was a tool to address larger evolutionary questions. For example, microbial systems were exploited to address questions addressing the predominance of the diploid phase [27], and the neutral theory of evolutionary change [28,29]. One question that continues to stimulate particular interest among evolutionary biologists, concerns the conundrum of the evolution of sex, and the potential to address it through experimental microbial systems. Theoretical arguments for the evolution of sexual reproduction abound, yet experimental

demonstration has remained elusive. *S. cerevisiae*, with both sexual and asexual cycles, has long been an enticing model organism for such studies among evolutionary biologists [30,31]. Yet, any demonstration of the advantage of recombination in an experimental setting, where populations are induced to go through the meiotic cycle, may be a result of general significance, yet of limited relevance to the understanding of the adaptive significance of sex in *S. cerevisiae*, as i) cells only enter the meiotic cycle when starved, ii) the meiotic cycle is significantly longer that the mitotic cycle, iii) and results in the formation of resting (asco) spores, which, iv) are highly inbred as mating typically occurs in the ascus where any of the four spores can mate with each other on germination as wild strains are homothallic. All these features of the life cycle of yeast in nature can be expected to severely attenuate any likely advantage of the sexual cycle.

5. Exploiting the power of the microbial system

The growing body of biochemical and physiological information on the model microorganisms, *E. coli*, *Salmonella typhimurium* and *S. cerevisiae* together with the application of powerful techniques of genetic manipulation and mapping, developed in the 1970s and 1980s, allowed the genetic analysis of evolving microbial populations with a resolution that was not hitherto possible. Thus, in the 1980s evidence began to accumulate that monocultures of asexual microorganisms growing in a simple unstructured environments with a single limiting resource (glucose-limited continuous culture) evolved to become stably polymorphic after as little as ~100 generations [32]. These findings were of interest to evolutionary biologists as they appeared to contradict two basic tenets of evolutionary theory: the classical model of the evolution of asexual organisms [33] and the so-called competitive exclusion principle [34]. The genetic changes associated with these stable polymorphisms and the mechanisms by which they are maintained were analyzed with increasing resolution in several subsequent publications [35–37]. Work by Ferenci and colleagues [*e.g.*, 38] has also enhanced our understanding of the molecular, biochemical and physiological bases of adaptation when asexual species such as *E. coli* evolve in simple unstructured environments, showing, for example, how mutations in the global stress-response regulator *rpoS* arise repeatedly and increase the capacity of slow-growing cells to acquire limiting resources [39]. Later, detailed analyses of these populations further revealed that epistatic interactions among highly pleiotropic mutations in *rpoS*, *mglD*, *malT*, and *hfq* drive the emergence and coexistence of multiple *E. coli* lineages in simple glucose-limited environments [40].

In a similar fashion early observations of large-scale genomic change occurring in continuous cultures of *S. cerevisiae* [41], subsequently could be analyzed at a high resolution, leading to an understanding at the molecular level of the underlying ectopic recombination events leading to the rearrangements [42].

6. Impact of the revolutions in sequencing and other biotechnologies to EME

Beginning in the 1990s, and initially driven by the Human Genome Project, successive revolutionary advances in biotechnologies, particularly in DNA sequencing, sequence analysis and database management transformed the field of experimental microbial evolution. Not only did they allow existing issues in microbial evolution occurring both in the laboratory as well as in nature to be examined in ever greater detail, but perhaps more significantly they allowed addressing a completely different set of questions — which can be considered fundamental to our understanding of the evolutionary process.

Given their small size and relative simplicity, the first genomes to be completely sequenced were microbial; the bacteriophage lambda [43], *Mycoplasma* [44], *Haemophilus* [45] and *E. coli* [46]. Further, as a result of a remarkable community effort, *S. cerevisiae* became the first eukaryotic genome to be completely sequenced [47], and subsequently, as a testing bed for the development of sequence-based technologies by which genomes, transcriptomes [48,49], protein–protein [*e.g.*, 50] and protein–DNA interactions [*e.g.* 51,52] could be systematically and comprehensively investigated in high-throughput fashion. Hence microbial evolutionary biologists were first in line to take advantage of this newly acquired wealth of data. Technologies such as microarrays quickly found application in the study of evolving microbial systems leading to the discovery, for example, that replicate yeast populations experimentally evolved under nutrient limitation independently converge on a physiological phenotype and shared transcriptional program that differs dramatically from their common ancestor [53]. This same tool has been employed to interrogate both experimentally evolved and naturally occurring microbial genomes for amplifications, deletions, insertions, rearrangements and base-pair changes [54], and continues to be used to assay global patterns of gene expression in a variety of experimentally evolved species including *E. coli* [55–57 and refs. within, 58, 59], *Myxococcus xanthus* [60], *Lactococcus lactis* [61] and *S. cerevisiae* [62,63].

Over the past decade, continued refinement and widespread development of new post-Sanger sequencing technologies have resulted in a reduction in DNA sequencing costs by orders of magnitude. While these new technologies have opened the door to the possibility of "personalized" medicine, they have also revolutionized the field of experimental microbial evolution, making it possible to address experimentally, fundamental questions in evolution not hitherto possible, such as how founder effects, historical contingency, pleiotropy and epistasis control the tempo and trajectory of evolutionary change. Forty years ago Lewontin [64] argued that the major goal of evolutionary biology was to understand the adaptive significance of genetic variation observed; a goal whose full achievement requires a detailed, molecular understanding of how different genotypes map onto biochemical, physiological and fitness phenotypes. Whole genome sequencing, used in conjunction with experimental microbial evolution, has shown exceptional promise for helping to achieve these goals [65]. Proof-of-concept for this general approach was first reported in 2005 by Shendure et al. [66], who used polony sequencing to discover five SNPs and two deletions that arose in a tryptophan-defective *E. coli* strain following 200 generations of coculture with a tryptophan prototroph. A year later whole genome sequencing was used to characterize terminal clones isolated from replicate *E. coli* populations which evolved by serial dilution in glycerol minimal medium [67]. This latter study uncovered remarkable instances of parallel evolution in the gene that encodes the first step in glycerol metabolism, *glpK* as well as in *rpoB* and *rpoC*, genes that encode two of the major subunits of RNA polymerase. Also in 2006, whole genome resequencing was used in conjunction with experimental evolution to uncover the genetic bases of a complex phenotype underlying "cheating" and "cooperation" in the social bacterium *M. xanthus* [68]. Since the publication of these landmark papers, numerous studies have appeared that, in various ways, with various species have markedly enhanced our understanding of the topology of the genotype–phenotype map, uncovering, for example genetic bases of adaptation to continuous nutrient limitation in yeast and *E. coli* [62,63,69,70], the genetics underlying the emergence of stable polymorphisms in continuous (chemostat) [3736,40] and discontinuous (serial dilution) cultures [71], and the molecular basis for an evolutionary innovation that provides cells access to a previously inaccessible food resource [72].

Whole genome sequencing of individual evolved clones and their ancestors, followed by detailed physiological studies, allelic replacement experiments and fitness assays, has dramatically improved our understanding of the genotype–phenotype map. However, this approach alone has done little to illuminate the structural and dynamic features of the adaptive landscape, *sensu* Wright [73] and how genetic opportunities and constraints shape that landscape over time. Recent advances in meta-genomics have enabled comprehensive analyses of allele frequency dynamics in experimental populations, making it possible for the first time to illustrate once-theoretical "Muller" [33]

diagrams with actual data [*e.g.*, 74,75]. These studies have revealed a "post-Mullerian" complexity that previously could only be postulated [76]. The first of these investigations tracked the evolutionary dynamics of novel alleles arising in populations of three model species cultured under three different conditions: *E. coli* grown in a fluctuating environment limited on two resources [77], yeast grown in either a fluctuating environment [78] or in a constant environment limited on one resource [79], and *Burkholderia cenocepacia* grown in biofilms [80]. All of these studies uncovered striking parallelism at the genetic and phenotypic levels: in the *E. coli* study two specialist ecotypes repeatedly evolve, each *via* a common set of genetic changes; in yeast, dysregulation of nutrient signaling pathways repeatedly arises, albeit by diverse genetic mechanisms; and in *Burkholderia*, biofilm specialists repeatedly arise whose adaptations converge on altered regulation of a limited set of pathways controlling, for example, metabolism of cyclic diguanosine monophosphate and polysaccharide biosynthesis. Salient features of evolving populations in each of these experiments are genetic hitchhiking and clonal interference, which lead to the accumulation of neutral or even slightly deleterious alleles in adaptive lineages and to the coexistence of multiple lineages owing to the fact that their relative fitnesses *in situ* preclude any one of them from going to fixation. These studies very likely will serve as models for future investigations, as each sought to discover not only allele frequency dynamics over an experimental time course, but also to discern the linkage relationships among these alleles by sequencing individual adaptive clones and their ancestors. For example, Payen et al. [81] recently employed this approach to discover the evolutionary paths by which yeast adapt to continuous sulfate limitation.

7. Recap and prospectus

The field of experimental microbial evolution has progressed, in its short history, by the activities of practitioners in two disparate sub-disciplines of Biology – biochemistry/physiology and evolutionary biology – whose early work often failed to acknowledge the contributions of each other. Classical evolutionary biologists initially did not recognize either the power of the microbial system or the intrinsic interest in microbial evolution, as illustrated by Julian Huxley (at the time, by no means an apostate in this regard) who wrote in 1942 [82] that bacteria

> "*have no genes in the sense of … hereditary substance. Occasional 'mutations' occur we know, but there is no ground for supposing that they are similar in nature to those of higher organisms… We must, in fact expect that the processes of variation, hereditary and evolution in bacteria are quite different from the corresponding processes in multicellular organisms.*"

A lack of interest by both sets of parties in each other's discipline frustrated early workers in EME, and stimulated the writing of a proposal by Bruce Levin to hold a biennial Gordon Research Conference on Microbial Population Biology. The first such conference was held in 1985; attendees at early meetings included such luminaries from the two sub-disciplines as John Maynard-Smith, James Crow and Allan Campbell, and did much to catalyze intercourse between evolutionary biologists on the one hand and molecular microbiologists on the other. The field experienced what could be termed "hybrid vigor", as mainstream evolutionary biologists came to realize that some of the "big" questions in evolutionary biology could only be addressed in microbial systems, and microbiologists trained as biochemists and physiologists and came to appreciate that, in Dobzhansky's words [83] "*nothing in* [micro]*biology makes sense except in the light of evolution*". Evidence of the convergence of the various areas in Biology may perhaps be seen in a rewording of Dobzhansky's perceptive quote to be, variously, "nothing in evolution makes sense except in the light of population genetics [84], ecology [85] or DNA [86]".

Advances in DNA sequencing and sequence analysis are rapidly changing the landscape of EME. But it also appears likely that as this field advances it will be further enriched by workers having expertise in systems biology and in structural biology, and experienced in future advances in biotechnology, such that we may be expected to learn, for example, how particular adaptations are forged through molecular–molecular interactions, as well as by subcellular structural constraints. Continued synergies with workers in mainstream evolutionary biology will be encouraged by the biennial Gordon Research Conferences in Microbial Population Biology as well as with the recently inaugurated biennial American Society of Microbiology Small Conference on experimental microbial evolution.

Acknowledgments

The authors thank G. and L. Parrot, and G. Sherlock, for their helpful comments on the manuscript. Preparation of this manuscript was supported in part by NASA Exobiology (NNX12AD87G) grant to FR. This paper was stimulated in part and enhanced by the many remarkable papers contributed by colleagues attending the first American Society of Microbiology Small Conference on Experimental Microbial Evolution, held June on 19–22, 2014 in Washington, DC.

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