Evolution of Cross-Feeding in a Simple Bacterial Community: Genetic Determinants

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Abstract

Syntrophic interactions, like cross-feeding, are ubiquitous in the microbial world and occur between different species and between different ecotypes of the same species. We seek to understand the conditions that favor intraspecific syntrophy using experimental evolution. Previous experiments led us to hypothesize that a limited number of mutations, perhaps only two, favor the emergence of cross-feeding lineages from a single ancestral clone. These mutations result in the overproduction of, and restricted access to, overflow metabolites. To test this hypothesis, we used site-directed mutagenesis to introduce the two mutations into an otherwise wild-type *Escherichia coli* strain, K12 MG1655. This genetically engineered strain was then evolved under glucose limitation as was done in previous studies where cross-feeding was observed. The resulting experimental populations are currently being analyzed for the emergence of inter-clonal cross-feeding. The discoveries made possible by pursuing this line of inquiry promise to enhance our understanding of how metabolically complex communities emerged early on in the history of life on Earth and illuminate why certain mutational trajectories are followed in polymorphic diseases like cancer.

Introduction

The chief tenet of evolutionary theory is that all the complexity and diversity of life arose from the same primordial origins. How and why the first primitive cells began to collaborate remains something of a mystery, as we have yet to fully understand what makes it favorable for individual cells to shift their life history from one of solo competition, to one of symphonic cooperation. To better understand which environmental and genetic determinants may have triggered this evolutionary transition, we can use experimental evolution to test how simple organisms like *E. coli* form cooperative communities. Prior research has shown that a bacterial population founded by a single clone rapidly increases in both size and genetic diversity, even in a constant environment (8). Still unknown are the genetic factors that determine what evolutionary trajectory a given population may follow. Unveiling the mechanisms by which clonal diversity is produced and maintained will improve our understanding of how clones evolve under stress, communicate, and coexist in both cancer and infectious disease.

We seek to further elucidate how a stable cross-feeding bacterial consortium consisting of multiple *E. coli* clones can arise from a single common ancestor (4). Evolution experiments in which this result has been observed are dominated by evolved clones that differ from typical "wild type" *E. coli* (6). For example, in one such experiment this clone carries mutations that result in high-affinity glucose uptake when glucose is limiting, including some that compromise overall metabolic efficiency. These mutations favor fermentation, suppress TCA cycle activity and respiration, and create redox imbalance. We predict that this metabolic shift drives the evolution of cross-feeding, and indeed, production of certain overflow metabolites helps regenerate NAD+ for continued glucose assimilation. The goal of this study is to determine which genetic factors favor evolution of clonal reinforcement, a process rooted in the establishment and partitioning of metabolic niches as well as in the evolution of cellular differentiation.

Background

Structure of the Consortia

In 1987, Helling and colleagues experimentally evolved *E. coli* JA122, under continuous glucose (0.0125% w/v) limitation at constant temperature (30°C) and growth rate (D=0.2 hr^{-1}) (4). After 765 generations in a chemostat bioreactor, researchers observed formation of a four-membered consortium that had evolved from a common ancestral strain. Consortium members differed genotypically and phenotypically and could be cultured in isolation as well as in co-culture. The evolved clones were shown to interact biochemically via clonal reinforcement, a simple form of collaborative metabolism (6). Rosenzweig et al. showed that this community or consortium was composed of a numerically dominant strain that avidly, but inefficiently consumed the limited carbon source, glucose, and other strains that consumed a variety of its waste products including acetate (8). These interactions served to increase the fitness of the entire consortium relative to the common ancestor (10). With the advent of advanced recombinant methods and Next-Generation sequencing, our ability to uncover the conditions required for such an evolutionary event to occur has markedly expanded. Recent papers by Kinnersley et al. and Gudlej et al. led us to hypothesize that a cross-feeding consortia is more likely to arise under certain genetic conditions (6, 7, 2).

Identifying Potential Genetic Factors

The first genetic factor we selected was originally identified by Treves et al. (9). This study showed that the ancestral strain JA122, and close relatives of that strain, were prone to acetate overflow during fermentation due to a point mutation upstream of the acetyl co-A synthetase gene (*acs*). This ancestral mutation dysregulates the pathway by which *E. coli* imports acetate, a major fermentation by-product. The presence of residual acetate in a low-nutrient environment may select for mutants that can exploit this new niche. Kinnersley et al. later found that mutations that increased glucose-scavenging capacity (e.g., in the regulation of LamB and MglABC expression) arose early on in the original experimental evolution study and were strongly favored (6, 4). These combined into a single lineage, CV103, which avidly consumes glucose but does so fermentatively, releasing even more overflow metabolites (7). Kinnersley et al. later discovered a second possible key mutation in this lineage, a missense single nucleotide polymorphism (SNP) in *lpd* which encodes for lipoamide dehydrogenase. This enzyme is a constituent of three multi-enzyme complexes (7, 6). The particular *lpd* mutation observed was shown to slow growth rate, increase flux to acetate and repress the TCA cycle. Thus, we hypothesize that the ancestral *acs* mutation in conjunction with the evolved *lpd* mutation are essential to creating a chemically complex environment that supports the emergence of adaptive mutants having the capacity to utilize secondary resources.

Methods and Materials

Plasmids and Strains

E. coli strain K12 MG1655 was revived from previous lab stocks acquired from Dr. Margie Kinnersley at the University of Montana. Other *E. coli* strains, JA122, CV101, and CV103 were revived from glycerol stocks stored at -80°C.

A temperature sensitive helper plasmid, pSLTS equipped with the λ-red recombinase system was used to increase homologous recombination events during mutagenesis and was obtained from Addgene. Components of the mutation template plasmid (MTP), designed to carry the mutation cassette, were obtained from the Copley lab at the University of Colorado Boulder (5).

Mutations

Both mutations were identified based on genomic data provided in Kinnersley et al 2014 (7).

The first mutation of interest was identified in the Crp binding site of the *acs* gene in the ancestral strain of the previously observed cross feeding community. The point mutation is located at base pair 4287464 of the whole genome sequence of JA122 and is a transversion from a T to an A. This missense mutation results in a downregulation of the encoded enzyme, acetyl-CoA synthetase which leads to an impaired ability to metabolically recycle acetate.

The second mutation was identified in the evolved glucose expert, CV103. The mutation is a C to A transversion which results in an amino acid change from phenylalanine to leucine at location 76 in the coding sequence of *lpd*. This (F76L) substitution occurs in the N-terminal portion of the translated protein, specifically in the FAD binding domain, likely affecting electron transfer from the reduced co-factor $FADH_2$ to NAD+. This mutation represses TCA cycle activity, which in turn increases the fermentative output of acetate.

Mutagenesis

Single mutant strains (K12 acs^- and K12 lpd^-) were engineered by collaborator Juhan Kim at the University of Colorado Boulder, according to a previously developed scar-less site-directed mutagenesis protocol (5). **Figure 1** illustrates this method, which utilizes λ-red recombinase provided on a helper plasmid to facilitate homologous recombination of a linear mutation cassette onto the host chromosome. The integrated mutation cassette is equipped with an antibiotic selection marker that can be removed by a highly specific I-*Sec*I cut site to generate a scar-less point mutation at a specified site in the genome. After receiving the single mutants, we generated a double mutant strain (K12 acs^- lpd^-) using this same protocol by introducing the *lpd* point mutation into a K12 acs^- mutant strain.

Characterization

Both single and double mutant strains were characterized under glucose limitation along with the wild-type K12strain, ancestral strain JA 122, and evolved clones CV103 and CV101. Growth rate analysis carried out in Davis minimal media containing salts, vitamin B1, and 0.015% glucose. Specific growth rate was calculated in a 24-well microtiter plate by measuring Absorbance via spectroscopy at λ=550nm. Maximum growth yield was analyzed after 48 hours of growth in batch culture (50ml culture in a 250ml Erlenmeyer flask), also by measuring absorbance at λ=550nm.

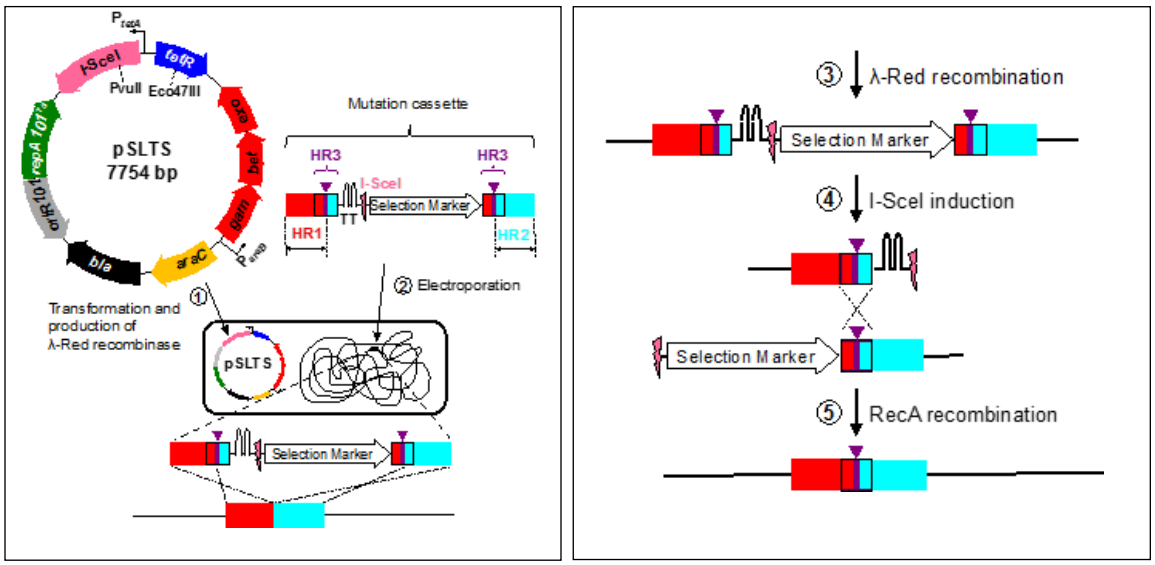

Figure 1. Schematic of Scar-less site directed mutagenesis adapted from Kim et al 2014
A temperature sensitive helper plasmid, pSLTS, is transformed into target cell along with the linear mutation cassette. Homologous arms, HR1 (red) and HR2 (blue) are regions that are complementary to the target cell's genome around the mutation site. HR3 overlaps with HR1 and HR2 and houses the point mutation. The Mutation cassette is equipped with chloramphenicol resistance as a selection marker. After selection of successful transformants the selection marker is removed via I-SceI cleavage. Homologous recombination then joins HR1 and HR2 and the desired mutant sequence is obtained.

Evolution

The double mutant (K12 *acs*$^{-}$, *lpd*$^{-}$) was experimentally evolved in glucose-limited chemostat s in parallel, using the wild-type K12 as a control. Evolution experiments were conducted in triplicate for more than 200 generations. Medium, consisting of Davis minimal salts, 0.0125% glucose w/v, and vitamin B1, was supplied at a constant dilution rate of 0.2 h^{-1} to each chemostat; temperature was maintained at 30°C, as per previous work (4). Populations were sampled every other day and plated on agar containing acetate, or glucose as a preliminary screen of heterogeneity in colony morphology within the population, as previous work had shown that glucose specialists formed small colonies, whereas those that could also feed on actetate formed large colonies (4, 8, 9). Every other day 1.5ml population samples were placed in 20% glycerol then archived at -80°C for later analysis.

Results

Characterization of the engineered mutants gave no evidence that the *acs* and *lpd* mutations significantly altered the growth rate phenotype of K12 **(Table 1)**. This was somewhat surprising as we regularly observed that the K12 *lpd*$^{-}$ mutant took about twice as long as wild-type *E. coli* (48 hours) to grow on complete medium agar plates. However, the mutations do appear to have negatively impacted cell yield. Additional experimental replicates will be required to rigorously test the significance of these cell yield results.

Strain	Growth Rate (0.015% glucose)		Yield at 48h
	μ hr^{-1}	±SEM	Relative to JA122
JA 122 (*acs*⁻, *lpd*⁺)	0.38 [a,b]	0.01	1.00
CV 103 (*acs*⁻, *lpd*⁻)	0.44 [a,b]	0.04	0.85
CV 101 (*acs*⁺, *lpd*⁺)	0.48 [a]	0.07	1.10
K12 WT (*acs*⁺, *lpd*⁺)	0.36 [b]	0.03	0.95
K12 (*acs*⁻, *lpd*⁺)	0.47 [a,b]	0.02	0.85
K12 (*acs*⁺ *lpd*⁻)	0.39 [a,b]	0.01	0.88
K12 (*acs*⁻, *lpd*⁻)	0.35 [b]	0.00	0.76

Table 1. Growth in 0.015% glucose batch culture Davis MN. Maximum specific growth rate (μ) is presented as the mean of triplicate experiments. Cell yield was estimated as A_{550} following 48 h of growth in 250ml flask (50ml culture). A one-way ANOVA showed significant differences among mean growth rates ($p < 0.05$). Superscripted letters denote significant differences among means (Tukey post-hoc comparisons, experiment-wise $\alpha = 0.05$). Results do not support a growth rate difference between wild-type and mutant strains.

In order to determine whether any strain was capable of sustaining cross-feeding we ran a simple experiment testing the growth of a known acetate "expert," CV101, in the spent medium of our different strains **(Table 2)**. As expected from earlier studies (Helling et al. 1987) CV101 was able to grow in the spent medium of CV103. In addition, CV101 was able to grow in spent medium of JA122 *acs*⁻, K12 *acs*⁻, and K12 *lpd*⁻, *acs*⁻. These results show that CV101 was only able to grow in the spent media of strains that carried the *acs* mutation, which supports our hypothesis that this point mutation is an important factor in the emergence of cross-feeding.

Strain	Growth of CV101 in spent media
JA 122 (*acs*⁻, *lpd*⁺)	Yes
CV 103 (*acs*⁻, *lpd*⁻)	Yes
CV 101 (*acs*⁺, *lpd*⁺)	No
K12 WT (*acs*⁺, *lpd*⁺)	No
K12 (*acs*⁻, *lpd*⁺)	Yes
K12 (*acs*⁺ *lpd*⁻)	No

Table 2. Growth of CV101 in spent media. Spent media was collected from 48h batch cultures grown in Davis minimal media (0.025% glucose w/v). Each experiment was run in replicates of six with CV101 being inoculated into each at a consistent dilution. CV101 was inoculated into its own spent media as a control.

Experimental evolution was conducted for over 200 generations in constant culture chemostats. Throughout this experiment samples were collected of both the evolving populations and their spent media. Analysis of the carbon sources present in the spent media **(Figure 2)** show that acetate remains only in the spent medium of K12 *acs*$^{-}$ *lpd*$^{-}$ founded populations. Before the experiment reached 100 generations, all three replicates show depletion of the secondary carbon source, suggesting that a revertant phenotype may have arisen in all three populations.

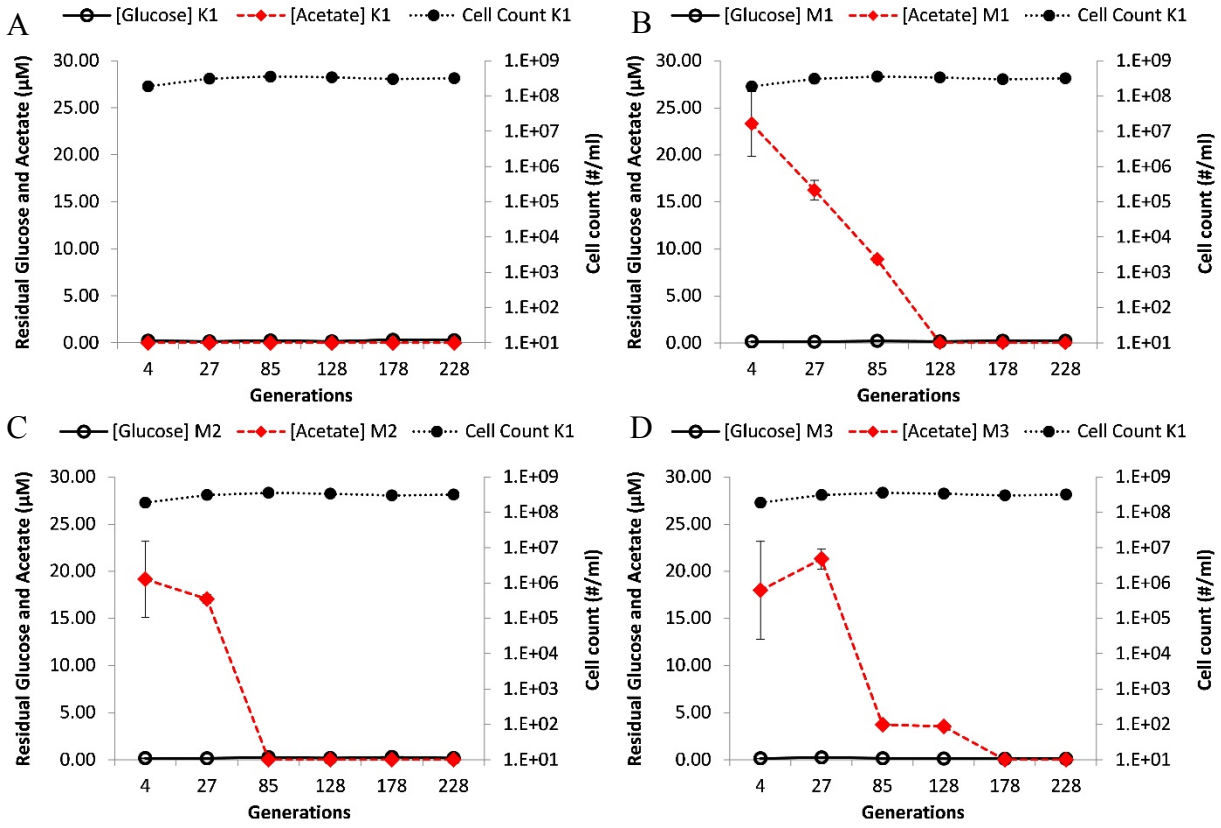

Figure 2. Presence of carbon sources in spent media throughout experimental evolution. (A) Representative of control populations, residual acetate is never observed during experimental evolution, likely because wild-type K12, with its fully functional Acs protein, can consume any acetate produced. Acetate overflow may be limited, relative to the mutant K12 *acs*$^{-}$*, lpd*$^{-}$*,* due to a functional Lpd protein that maintains TCA cycle flux even in the presence of limiting glucose. (B, C, D) Populations founded by K12 *acs*$^{-}$*, lpd*$^{-}$, all initially exhibit ~20mM residual acetate. However, within ~100 generations all acetate has been depleted suggesting that a revertant *acs* phenotype has emerged in the population, as hypothesized.

Discussion

Surprisingly, when glucose is *non-limiting* neither the missense mutation in Lpd (F76L) nor the CRP-binding site mutation -93 bp upstream of Acs significantly impact the maximum specific growth rate (μ_{max}) of K12. However, preliminary data suggest that these mutations may reduce yield, as would be expected if strains were respiratory-deficient. Additional replicates are required to validate this observation. Further analysis of strains' spent media showed that the K12 *lpd*$^{-}$ *acs*$^{-}$ double mutant can support growth of acetate 'experts' like CV101, which constitutively overexpresses acetyl CoA synthetase (8). This result is important because it demonstrates that without the *acs* regulatory mutation *E. coli* can still quickly re-assimilate low

levels of acetate. Based on these findings we chose to carry out evolution experiments using K12 *lpd*$^{-}$ *acs*$^{-}$, with the K12 wild-type serving as a control.

Appreciable levels (~20mM) of residual acetate were observed in all three chemostats founded by the double mutant but not in chemostats founded by K12 (**Figure 2**). Therefore at least initially, a secondary carbon source was available to support the growth of *de novo* mutants that could acquire the ability to access it. The fact that this resource was depleted by generation 100 suggests that such de novo mutants did in fact arise. Plating population samples on acetate minimal agar we observed a mixture of Large and Small colony variants, exactly as originally reported by (4) **(Figure 3)**, further supporting the hypothesis that cross-feeding may have emerged in populations founded by double mutants. We are in the process of Sanger sequencing the *acs* coding and regulatory regions of these Large and Small colony variants, anticipating the discovery of mutations that *restore* Acs activity in the former, but not in the latter. Finally, we will perform whole-genome, whole-population sequencing on population samples at 0, 50, 100, 150 and 200 generations to determine changes in the frequency of *de novo* mutations that could be expected to impact acetate metabolism.

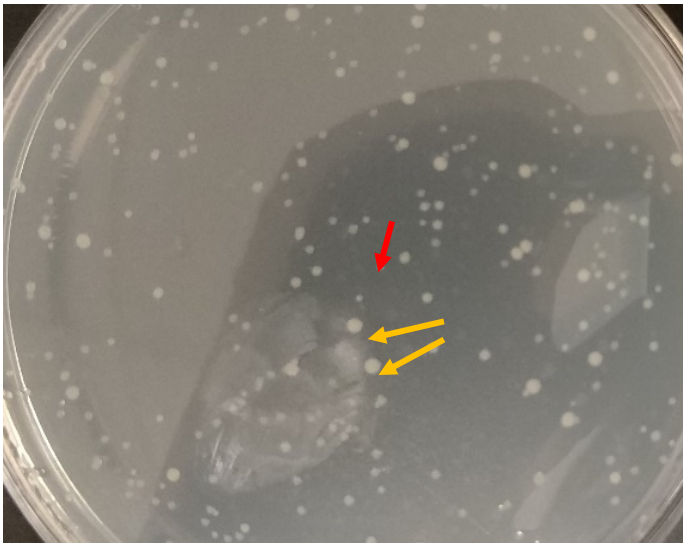

Figure 3. Preliminary screen for de novo acetate experts. In order to determine whether acetate experts arose during the course of evolution we plated population samples on agar plates amended with 1mM acetate as the sole carbon source. Large colony variants (LCV) are reminiscent of colonies formed by the acetate specialist CV101 (yellow arrows), while small colony variants are reminiscent of those formed by the glucose specialist, CV103 (red arrow) (4, 6). CV103 is an *acs*$^{-}$ mutant; CV101 is *acs*$^{+}$ (**Table 1**).

After completing our evolution experiments we noticed that biofilms had formed in the tubing that fed fresh medium to each of our chemostats. We confirmed that in each case the biofilms were not contaminants but consisted of the *E. coli* founder strains. We plan to sequence these biofilm *E. coli* and account for their presence in our experimental populations. Analysis of the phenotype of this biofilm and the frequency of its constituents will determine whether its presence will inhibit our ability to draw strong inferences from this study. It is encouraging to note that in a parallel study by Kinnersley et al. (In preparation) lineages of biofilm origin made up about 5% of each evolving chemostat population, and in no case strongly impacted the evolutionary trajectory of planktonic cells in the chemostats.

In conclusion, the mutants engineered here provide useful tools for addressing questions about how cross-feeding emerges in a simple constant environment. The implications of our findings promise to shed light on how early microbial life on Earth transitioned from genetically simple to genetically diverse communities, and on how such transitions manifest as metabolic interactions among clones in cancer and chronic infectious disease.

Acknowledgements

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