Long-Term Diversity and Genome Adaptation of Acinetobacter baylyi in a Minimal-Medium Chemostat

Nadia Jezequel¹,², Marco Cosentino Lagomarsino¹,³,⁴, Francois Heslot¹,², and Philippe Thomen¹,²,*
¹Université Pierre et Marie Curie, Paris, France
²Laboratoire Pierre Aigrain, Ecole Normale Supérieure, CNRS (UMR 8551), Université P. et M. Curie, Université D. Diderot, Paris, France
³Génophysi/Genomic Physics Group, CNRS (UMR 7238) “Microorganism Genomics,” Paris, France
⁴Dipartimento di Fisica, Università degli Studi di Torino, Torino, Italy
*Corresponding author: E-mail: philippe.thomen@upmc.fr; thomen@lpa.ens.fr.
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Abstract

Laboratory-based evolution experiments on microorganisms that do not recombine frequently show two distinct phases: an initial rapid increase in fitness followed by a slower regime. To explore the population structure and the evolutionary tree in the later stages of adaptation, we evolved a very large population (~3 x 10¹⁰) of Acinetobacter baylyi bacteria for approximately 2,800 generations from a single clone. The population was maintained in a chemostat at a high dilution rate. Nitrate in limiting amount and as the sole nitrogen source was used as a selection pressure. Analysis via resequencing of genomes extracted from populations at different generations provides evidence that long-term diversity can be established in the chemostat in a very simple medium. To find out which biological parameters were targeted by adaptation, we measured the maximum growth rate, the nitrate uptake, and the resistance to starvation. Overall, we find that maximum growth rate could be a reasonably good proxy for fitness. The late slow adaptation is compatible with selection coefficients spanning a typical range of 10⁻³–10⁻² per generation as estimated by resequencing, pointing to a possible subpopulations structuring.

Key words: Acinetobacter, experimental evolution, resequencing, nitrogen limitation, chemostat, large population.

Introduction

Understanding adaptation is important in a wide number of biological, ecological, and technological contexts (Toft and Andersson 2010; Jackson et al. 2011). In this framework, controlled laboratory evolution experiments performed on large populations of bacteria can explore adaptation under well-defined selection pressure (Buckling et al. 2009; Conrad et al. 2011; Hindré et al. 2012). Through phenotypic characterization and sequencing, these experiments give a quantitative insight into the genomic adaptation of microbial populations (Barrick and Lenski 2009; Barrick et al. 2009; Tenaillon et al. 2012).

Possibly, one of the best examples of the impact of this approach concerns fundamental ecology. As a result of the “competitive exclusion principle” (Gause 1934; Hardin 1960), it was previously believed that diversity cannot be created and maintained in simple, spatially, and temporally homogeneous environments containing a single resource. The recent in vitro evolution experiment of Maharjan et al. (2006, 2012) has disproven the competitive exclusion principle for large populations in the laboratory and demonstrates that a clonal population of Escherichia coli evolving in a chemostat can diversify into a number of subpopulations.

The mechanisms responsible for this diversity are unclear and include convergent evolution and regulatory degeneracy (Gresham et al. 2008; Wang et al. 2010), cross-feeding (Rosenzweig et al. 1994; Friesen et al. 2004), and frequency-dependent selection (Levin 1988; Doebeli and Ispolatov 2010). Beardmore et al. (2011) recently revealed a fourth mechanism, which explains the emergence and maintenance of diversity in simple environments by the existence of metabolic and physiological trade-offs. A fifth possibility is related to the fact that for such large population-size experiments in the absence of recombination, many beneficial mutations compete to drive adaptation, through “clonal...
interference” (Gerrish and Lenski 1998). A recent laboratory evolution study (Hughes et al. 2012) found stable competition between coexisting hosts with different plasmid genotypes in plasmid–host adaptation dynamics, suggesting that this diversity plays a role in the evolution of the persistence of drug resistance. Despite recent theoretical and experimental advances (Desai and Fisher 2007; Desai et al. 2007; Park and Krug 2007; Brunet et al. 2008; Good et al. 2012), this regime of adaptation is not completely understood. For example, many open questions concern the dynamics of the advantage (Barrick et al. 2009), the role of epistasis (Chou et al. 2011; Khan et al. 2011), and the structuring of a population into subpopulations of competing clones (Maharjan et al. 2007). A recent study (Maharjan et al. 2012) used large-scale genomic and phenotypic analysis of the chemostat evolution data to identify the extent of diversification. They placed their results in the context of the above theoretical mechanisms of evolutionary divergence and proposed that diversity is unlikely to be explained by any one of the available theoretical models.

A criticism of the earlier study of Maharjan et al. (2006) concerned the relatively short evolutionary time scale of the experiment (26 days), which might lead some to speculate that running similar experiments for longer times would destroy the observed diversity (Conrad et al. 2011). Here, we present an evolution experiment with a different organism, Acinetobacter baylyi, that lasted 124 days (~2,800 generations), and we report stable and possibly increasing diversity that allows for an analysis of genome adaptation.

We constructed a chemostat culture using a strain of A. baylyi growing in a minimal medium, which in principle allows for a limited number of distinct ecological niches. The bacteria were grown with limited amounts of nitrate as the sole source of nitrogen. A high dilution rate (between 0.6 h⁻¹ and 0.9 h⁻¹), which should be the least favorable condition for evolution (Maharjan et al. 2012) was also used. Our system involves a unique growth medium supply and composed of interconnected alternating tandem chemostats (fig. 1), which prevents bacterial adhesion to the chemostat walls (de Crécy-Lagard et al. 2001). The main advantage of this setup (compared with serial dilution experiments) is that it

![Fig. 1](image-url)
allows for constant and controlled growth conditions involving very large population sizes. The main drawback is that it is cumbersome, and (with our resources) does not allow for replicate populations in parallel. However, this does not prevent the extraction of relevant information. Acinetobacter baylyi ADP1 has been previously sequenced and annotated (Barbe et al. 2004). We identified the mutations appearing during the experiment by genome resequencing at three different points in time and, by genotyping each mutation on isolated clones, resulted in the reconstruction of a partial population tree. These data provide approximate quantitative estimates of the selection coefficient of some mutations. We measured the associated changes in the maximum growth rate and found that the increase is consistent with the estimated selection coefficients.

**Materials and Methods**

**Bacterial Strain and Medium**

We used a strain of *A. baylyi* obtained from NCIMB (http://www.ncimb.com, last accessed January 3, 2013; NCIMB 11826). The minimal medium (pH 6.8) used in the evolution experiment comprised 50 mM potassium phosphate, 1 mM potassium nitrate, 3 mM potassium citrate, 9 mM citric acid, 1 mM magnesium sulphate, traces of elements (3 mM iron (III) chloride, 1 mM zinc sulphate, 1 mM cobalt chloride, 0.6 mM copper sulphate, 1 mM manganese sulphate, 1 mM sodium molybdate, 1 mM manganese chloride, and 1 mM calcium sulphate), and polypropylene glycol 2,000 at dilution 1:20,000 as antifoaming agent. For the initiation of the evolution experiment, the ancestral strain (ABWT) was plated, a single colony was in inoculated in the minimal medium and grown at 35°C until OD reaches 0.1–0.2. A volume of 50 ml of this culture was introduced in the reactor then completed by fresh medium. At various time intervals, samples were snap-frozen as stocks with 10% DMSO. Stocks were later plated and clones isolated. They are named ABGi, where G is the number of generations undergone by the bacteria before sampling, and i is a letter used to identify a clone.

**Experimental Evolution in a Chemostat**

Two interconnected reactors were used allowing aseptic periodic transfer from one reactor to the other, so that the reactor being not used for growth can be cleaned in situ, preventing long-term biofilm formation and bacteria evading dilution (de Crécy-Lagard et al. 2001). Transfer is made every week. The reactors (150 ml) are made of glass and have been assembled by glass blowing. One entry tube is used for the medium supply with its dripping opening inside the reactor and positioned above the liquid level of the culture. A compressed air input tube is plunged inside the reactor to the bottom. Air bubbles provide for aeration and mixing of the growth medium. An exit tube allows for both air and liquid outflow. Another entry is allowed for the periodic injection of a cleaning solution (1 M NaOH) within the reactor or for the transfer of the bacterial population before reactor cleaning. The reactors are connected to a unique growth medium supply via a flow rate controlling peristaltic pump (Masterflex). Temperature was regulated using a commercial PID regulator (ENDA ETC 442), maintaining the temperature at 35 ± 0.5°C. During the first 6 days, the dilution rate was kept below 0.5 h⁻¹, not to lose the population. Subsequently, it was kept constant between 0.6 h⁻¹ and 0.9 h⁻¹ (the reasons for these variations were both practical and related to experiments testing the maximum growth rate that occurs over the evolutionary time).

**Maximum Growth Rate Measurements**

The batch cultures were performed in a glass vessel presenting a lower part with a transparent square cross-section, so that an in situ optical density measurement could be performed. This system consisted of 1) a light emitting diode (LED) positioned in close proximity to one flat face of the reactor and emitting light inside the reactor, 2) a first photosensitive detector PD1 placed on the other side of the reactor and facing the LED, and 3) a second photodetector PD2 placed in close proximity of the light emitter. The current of the photo-detectors PD1 and PD2 were fed to a logarithmic-ratio amplifier, so as to yield a voltage related to the optical density of the culture. The reference value was obtained when fresh medium was first injected. The system yielded optical density measurements taken approximately every minute. The maximum growth rate in the batch mode for a set of clones was estimated from those measurements.

**Genome Resequencing**

We detected all events, including IS movements and DNA rearrangements, through genome sequencing. Genome resequencing of two endpoint clones was performed using Illumina platform by GATC-Biotech. The genome of the ancestral clone and the genomes of three sampled populations were resequenced using Illumina platform at GRCF HTS Center at Johns Hopkins University, Baltimore. Sequence alignment against the reference genome (*A. baylyi* ADP1) was performed using Moscow (http://bioinformatics.bc.edu/martha), last accessed January 3, 2013) or bowtie2 (Langmead et al. 2009). Visualization was performed using Tablet (Milne et al. 2010). Mutation detection was performed using S4MTools (Li et al. 2009) and gigaBayes (http://biomathematics.bc.edu/martha), last accessed January 3, 2013). The de novo genomic assembler velvet (Zerbino and Birney 2008) was also used. These tools are designed to extract allele frequencies and thus are also well adapted for the detection of mutation frequencies in mixed populations.
Sequencing and Reconstruction of the Population Tree

To study the mutational patterns and connect them with the evolutionary dynamics, we resequenced several samples: the ancestral clone (ABWT), two endpoint clones (AB2800a and AB2800b), and three samples of populations at three different time points (AB1900, AB2300, and AB2800) (We adopt the following notations: Clones/populations are labeled by the string AB followed by the number G and the label i, where G is the number of generations undergone by the clone or population and i is a tag to identify an isolated clone.). Sequencing of the ABWT genome revealed that it has differences with respect to the A. baylyi ADP1 genome (Barbe et al. 2004). The list of those differences is given as supplementary data, Supplementary Material online. The mutations that occurred during the experimental evolution from the ancestral ABWT clone are listed in table 1. To infer the order of appearance of the mutations and identify subpopulations carrying different mutations, we performed 378 short polymerase chain reactions (PCRs) on 87 isolated clones targeting the mutation loci, and sequenced the amplified fragments to test for the presence of the mutations, or tested for deletions or insertions by gel electrophoresis (see supplementary data, Supplementary Material online).

In total, we detected 23 mutations on 15 chromosomal loci, of which 10 and 11 are on the endpoint clones, which is in line with other evolution experiments with similar numbers of generations (Khan et al. 2011; Bachmann et al. 2012). Only four point mutations were found in genes, three of which were nonsynonymous. Remarkably (table 1), large events such as deletions (2), inversions (1), duplications (1), or insertion sequence (IS) transpositions (6) balanced in number small events such as single-nucleotide polymorphisms (SNPs) (11) and indels (2) (<30 bp) and seem to appear early on during the experiment. To our knowledge, this feature is not common to other experiments of this kind (Barrick et al. 2009; Wang et al. 2010; Tenaillon et al. 2012). Together, these data permitted the reconstruction of a partial evolutionary tree (fig. 2). To this end, an important feature is the detection of multiple co-occurring IS integration events just before 1,000 generations and concomitant with the disappearance of an IS present between ACIAD0290 and ACIAD0291 in ABWT (but not in ADP1). These two types of changes may be related to a single mutational event such as transposition of the latter IS to a new target. However, these changes may also involve two different mutational events. This target locus was variable among different clones as five different loci were identified. This gave birth to five detectable branches (named B1, B2, B3, B4, and B5). This IS insertion was used as a genomic marker to estimate the ratio of the five subpopulations in the sequencing of sampled populations.

The dynamics of branch B2 is remarkable. Figure 2 shows the near extinction of the branch over approximately 1,000 generations; at generations 980 and 1,100, one isolated clone out of 10 belongs to B2 (data not shown), whereas at generations 1,900 and 2,300, the sequencing of sampled populations indicates that the B2 branch occupies only approximately 1% of the population. However, the same branch increased to occupy nearly half the population at generation 2,800. Two mutational events appeared after 2,300 generations in the B2 branch and are seemingly associated with this uprise: an SNP upstream of ACIAD1029 and a deletion of 319 bp in the gltI gene (ΔgltI). The frequencies of these two genotypic features at the end of the experiment are 23% and 51%, respectively. Comparing these frequencies to the 47% of the B2 branch at the end of the experiment, we conclude that essentially all B2 bacteria carried the deletion, which is likely linked to a gain in fitness that allows the B2 branch to increase in relative proportion. This deletion event did not appear in the remainder of the population, suggesting that this event might be rare and/ or dependent on the mutational background. Notably, the locus of this deletion appears to be highly dynamic during the whole experiment as it involves two other different mutational events that are assumed to give a relevant advantage at two other different times: 1) an early inversion of 4,534 bp and 2) a point mutation (ACIAD2054) shared by several branches, with a frequency increasing from 26% to 91% from generation 1,900 to generation 2,800. However, it must be noted that also some genetic instability may increase the mutation frequency at that specific locus and that non-transitive interactions between subpopulations and negative frequency-dependent selection cannot be ruled out.

In brief, the combination of clonal and population sequencing with systematic mutation detection by PCR points to a complex scenario with highly dynamic coevolving subpopulations, even in the later stages of the experiment, where the main metabolic adaptation should have been already accomplished.

The Dynamics of Maximum Growth Rate Is Compatible with the Advantage Estimated from Population Sequencing

During the experiment, the dilution rate was typically kept constant (The dilution rate was below 0.5 h⁻¹ during the first 6 days.) between 0.6 and 0.9 h⁻¹. However, the dilution rate was sometimes transiently increased above the wash-out threshold to measure the maximum growth rate μ_max of the population, by real-time monitoring of optical density during the wash out. We assume that this transient increase in dilution rate did not interfere heavily with the population structure because it never washed out more than half of the population and lasted for approximately 1 h only (One may wonder whether this procedure could remove completely some low-frequency mutants. The probability of losing a
| Position on the ADP1 Chromosome | Gene - Region | Product | Mutational Event | Comment | \( G = 1,900 \) | \( G = 2,300 \) | \( G = 2,800 \) |
|--------------------------------|--------------|---------|-----------------|---------|----------------|----------------|----------------|
| 244223                         | glnK (a)     | Regulatory protein, for nitrogen assimilation by glutamine synthetase | C > T | 41 bp upstream of the start codon | ND | 20% | 41% |
| 389458                         | citA (a)     | Citrate proton symporter | T > C | Codon frequencies GAA (0.041) > GAG (0.014) | ND | ND | 29% |
| 1020409                        | AQAD1029 (b) | Putative lipoprotein | T > C | 100 bp upstream of the start codon | ND | ND | 23% |
| 1903797                        | AQAD1911     | Putative nitrate transporter transmembrane protein | G > A | 12 bp upstream of the start codon | ND | ND | ND |
| 1903816                        | AQAD1911 (a, b) | Putative nitrate transporter transmembrane protein | C > T | 31 bp upstream of the start codon | ND | ND | ND |
| 1903844                        | AQAD1911     | Putative nitrate transporter transmembrane protein | G > A | 59 bp upstream of the start codon | ND | ND | ND |
| 1903876                        | AQAD1911     | Putative nitrate transporter transmembrane protein | A > T | 91 bp upstream of the start codon | ND | ND | ND |
| 2042174                        | AQAD2054 (a, b) | Conserved hypothetical protein | T > C (S > P) | Nonsynonymous mutation in a gene yet cut by the inversion event | 26% | 65% | 91% |
| 2100627                        | AQAD2113     | Conserved hypothetical protein | A > G | 239 bp upstream of the start codon | 3% | 41% | 6% |
| 2790910                        | ftSH         | Cell division protein | G > A (H > Y) | Nonsynonymous | 33% | 57% | 10% |
| 2891560                        | qseB (a, b)  | Quorum sensing DNA-binding response regulator | G > A (S > F) | Nonsynonymous | 100% | 100% | 100% |
| **Small Indels**               |              |         |                 |         |                 |                 |                 |
| 1920605                        | AQAD1931     | Putative magnesium citrate secondary transporter | Ins A | 32 bp upstream of the start codon | ND | ND | 12% |
| 1249430                        | csrA (a, b)  | Carbon storage regulator | Δ8 bp | Last 15 amino acids (21% of the protein) modified | 100% | 100% | 100% |
| **Large Rearrangements**       |              |         |                 |         |                 |                 |                 |
| 1230681                        | AQAD1230 (a) | Putative transcriptional regulator | IS insertion | 79 bp upstream of the start codon (B1 branch) | 43% | 32% | 43% |
| 1230766                        | AQAD1230     | Putative transcriptional regulator | IS insertion | Within gene (B5 branch) | 9% | 7% | ND |
| 1230919                        | AQAD1230     | Putative transcriptional regulator | IS insertion | Within gene (B3 branch) | 32% | 53% | 10% |
| 1231207                        | AQAD1230 (b) | Putative transcriptional regulator | IS insertion | Within gene (B2 branch) | 1% | 0.5% | 47% |
| 1860246                        | AQAD1864–ACIAD1865 | Putative transcriptional regulators | IS insertion | Between genes (B4 branch) | 16% | 8% | ND |
Table 1 Continued

| Position on the ADP1 Chromosome | Gene - Region | Product | Mutational Event | Comment | G = 1,900 | G = 2,300 | G = 2,800 |
|----------------------------------|---------------|---------|------------------|---------|-----------|-----------|-----------|
| NA (a, b)                        | NA            |         | IS insertion     |         | 100%      | 100%      | 100%      |
| 1848721–1858316                  | ACIAD1848     | Putative phage replication initiation factor, putative phage-related protein, and putative phage replication initiation factor | Duplication | Spans ~10 kb | 100%      | 100%      | 100%      |
| 2041592–2046126                  | ACIAD2054, glut, glutK, glutJ, glutK (a, b) | Conserved hypothetical protein and glutamate/aspartate transport proteins | Inversion (4,534 bp) | Guts glutI and ACIAD2054; quenches glut operon | 100%      | 100%      | 100%      |
| 2046132–2046451                  | glut (b)      | Glutamate/aspartate transport protein | \(\Delta\)319 bp (named \(\Delta\)glut in the text) | Deletion in a gene yet cut by the inversion event | ND        | ND        | 51%       |
| 2664637–2726455                  | ACIAD2713–ACIAD2778 (a, b) | NA | S8 CDS deleted | ND        | 100%      | 100%      | 100%      |

Note.—List of detected mutations occurring in the experiment, separated by type (SNPs, indel, and large rearrangements). The last three columns indicate the frequency of each mutation at the three different sequencing times (G stands for the number of generations); 100 % frequencies are marked in boldface. NA, not applicable; ND, not detected (<1%). The letters (a) and (b) in the second column indicate if the mutation was detected in the endpoint clones AB2800a and AB2800b, respectively. Compared with other laboratory evolution experiments, there is a relevant number of large rearrangements, many of which appear early on and reach very high frequencies.

Thus, variations in mutation frequencies suggest that fitness contributions to complementarity between subpopulations of bacteria in a wash out of half the population can be estimated as 12%, let us consider a subpopulation of 20 individuals which corresponds to a frequency of approximately 0.0001%. We also measured the fitness of this subpopulation in culture plates, which corresponds to a frequency of approximately 0.0001%. We also measured the fitness of this subpopulation in culture plates, which corresponds to a frequency of approximately 0.0001%.
during the experiment. Even though the ratio between the dilution rate and $\mu_m$ is approximately 0.8, we cannot exclude that other ways of adaptation could be operative, in view of the fact that the chemostat dilution rate is set below $\mu_m$. To illustrate this aspect, let us compare two isolated clones, AB1100h and AB2800b. AB1100h was isolated and identified as belonging to the B2 branch. Its descendants are led to near extinction (between generations 1,900 and 2,300) but later acquire beneficial mutations that make them the largest subpopulation present at the end (fig. 2). It is thus expected that the fitness of AB2800b, an endpoint clone belonging to B2 branch, is larger than the fitness of AB1100h. Because of
One targeted trait could be nitrate uptake: Bacteria in a chemostat have to manage growth under limited nutrient availability. To measure how bacteria deal with nitrogen limitation, it is necessary to access their affinity for the limiting nutrient (nitrate). To do so, we grew a clone (isolated after 7 days) in a chemostat, up to an equilibrium for different dilution rates, and sampled extracts from the chemostat for each dilution rate. By a series of chemical reactions, we were able to estimate the nitrate concentration in each sample (supplementary data, Supplementary Material online). Plotting the nitrate concentration versus the dilution rate leads in principle to an estimate of the saturation constant. Unfortunately, the concentration was so low (<2 mM) that we failed to detect nitrate for all but the highest dilution rates (this value being likely above the wash-out threshold).

Thus 1) an increase in nitrate uptake after 7 days could not be measured in our experiment, but 2) we can conclude that bacteria evolved rapidly within 7 days (or were preadapted) to be able to capture >99.8% of the nitrate entering the chemostat, over a wide range of dilution rates. The following additional result is consistent with a good affinity for nitrate: measuring nitrate concentration at different times during growth in batch, we observed that cells grow exponentially until nitrate is no longer detectable, without a smooth transition between exponential and saturation phase (data not shown). In conclusion, if nitrate uptake optimization contributes to fitness, it does so only during the early stages of the experiment.

Adaptation might also consist of a better management of the low nitrogen level in the chemostat, that is, the evolved cells might be able to grow using lower amounts of nitrogen per cell. For example, an increase in protein turnover has been observed in *Pseudomonas putida* grown under...
Diversity in the Population Appears to Increase Slightly at Later Times

We first focus on the evidence for population diversity during the experiment. After the transposition of the IS that occurs after 1,000 generations, diversity is detected for the whole experiment, because three branches are still present at the end (fig. 2). Each branch evolved with both specific mutations (citA for B1, ACIAD1029 and Agl1 for B2, and ftsH for B3) and mutations that are shared with other branches (glnK and ACIAD2054). The latter likely appeared independently in different branches and are not related to horizontal transfer: In control experiments, the transformation frequencies of mutations. The indices calculated for generations 1,900, 2,300, and 2,800 are, respectively, 0.88, 1.04, and 1.34, suggesting a slight increase in diversity during the last 1,000 generations.

It is difficult to judge whether this diversity is large or small. Assuming constant additive s, standard population genetics theories (Desai et al. 2007, Brunet et al. 2008) would predict for our conditions the coexistence on the order of 10 subpopulation fitness classes. However, detailed information on linkage and precise measurements of fitness distributions would be necessary to connect fitness classes and mutation classes. Thus, given this large uncertainty, the observed diversity might well be in the predicted range.

Conclusion

To summarize, we performed a laboratory evolution experiment with bacteria growing in a minimal medium, which focused on its later stages. Sequencing of populations at different evolutionary times shows that the number and type of mutations are compatible with other laboratory evolution experiments, which are characterized by competition between variant clones (Barrick et al. 2009; Bachmann et al. 2012). Interestingly, we found many fixed large rearrangements compared with point mutations.

Also, we found significant and persistent diversity in a population with presumably the same ecotype (Maharjan et al. 2006; Woods et al. 2011). We thus report for the first time that diversity can be established and maintained in a very simple medium for long times, when the proxies for fitness appear to saturate. In this regime, where “adaptation” appears to have slowed down, we observe diversity that is stable or even increasing.

This observed long-term diversity can be due to different mechanisms, which were considered carefully in (Maharjan et al. 2012). Although it is beyond our scope to identify which of these mechanisms were active during our experiment, we can...
nevertheless formulate some qualitative and hypothetical considerations on the basis of our observations. Metabolic trade-offs (Beardmore et al. 2011) might be witnessed by the results of the biolog assays, where we observed changes in metabolic phenotypes that appeared a priori very distant from the expected target. An example of evolutionary convergence (Maharjan et al. 2012; Tenaillon et al. 2012) possibly can be pointed out by the insertion of an IS upstream or within a putative transcriptional regulator (ACIAD1230), which gave birth to four subpopulations (branches B1, B2, B3, and B5). At the same time, a fifth subpopulation (B4 branch) arose with the insertion of an IS between putative transcriptional regulators, in a totally different region (ACIAD1864–1865). The observation of a significant number of mutations in regulatory regions might also point toward a role played by convergence.

Maharjan et al. (2007) identified an evolved subpopulation of Escherichia coli specialized in the metabolism of by-products of other cells. In our case, reduced forms of nitrogen or amino acids could be cross-fed, so that a role for cross-feeding cannot be excluded. More generally, frequency-dependent selection could be active. Its effect has been observed at low dilution rates and could be ascribed to numerous ecological phenomena, including cross-feeding (Maharjan et al. 2012). In our experiment, the preservation of the B2 branch at low frequency during more than 400 generations could result from a frequency-dependent selection. This needs to be verified in future investigations.

Finally, in terms of basic parameters such as selection coefficient and population size, the results should be compatible with the regime where clonal interference can be observed (Park and Krug 2007; Hughes et al. 2012). However, clonal interference necessarily has to coexist with convergence (availability of alternative evolutionary pathways), and with the observed “diminishing returns” epistasis mechanism (Chou et al. 2011; Khan et al. 2011), according to which the advantage of beneficial mutations depends on their order of appearance.

Overall, we speculate that a possible way for the subpopulations to interact could be indirect through the effect of the limiting amounts of nitrogen in the medium. We explicitly tested for contributions to fitness that, if acquired by one subpopulation would affect the others, such as uptake and resistance to starvation. However, we were not able to identify such an effect. On the other hand, our results suggest that the maximum growth rate $m_{max}$ measured in batch might be a sufficiently good proxy for fitness per se, when compared with the selection coefficients estimated from resequencing data. This indicates that the observed subpopulation structure appears consistent with a regime in which competition between beneficial mutations could be relevant. Although both measurements are rough, we can surmise that if there are selected traits that are not recapitulated by $m_{max}$, their associated advantage cannot be very large.

**Supplementary Material**

Supplementary data are available at Genome Biology and Evolution online (http://www.gbe.oxfordjournals.org/).

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**Literature Cited**

Barbe V, et al. 2004. Unique features revealed by the genome sequence of Acinetobacter sp. ADP1, a versatile and naturally transformation competent bacterium. Nucleic Acids Res. 32:5766–5779.

Bachmann H, Starrenburg MJ, Molenaar D, Klerebezem M, van Hylckama Vlieg JE. 2012. Microbial domestication signatures of Lactococcus lactis can be reproduced by experimental evolution. Genome Res. 22:115–124.

Barrick JE, Lenski RE. 2009. Genome-wide mutational diversity in an evolving population of Escherichia coli. Cold Spring Harb Symp Quant Biol. 74:119–129.

Barrick JE, et al. 2009. Genome evolution and adaptation in a long-term experiment with Escherichia coli. Nature 461:1243–1247.

Beardmore RE, Gudelj I, Lipson DA, Hurst LD. 2011. Metabolic trade-offs and the maintenance of the fittest and the flattest. Nature 472:342–346.

Brunet E, Rouzine IM, Wilke CO. 2008. The stochastic edge in adaptive evolution. Genetics 179:603–620.

Buckling A, Maclean RC, Brockhurst MA, Colegrave N. 2009. The Beagle in a bottle. Nature 457:824–829.

Chou HH, Chiu HC, Delaney NF, Segra D, Marx CJ. 2011. Diminishing returns epistasis among beneficial mutations decelerates adaptation. Science 332:1190–1192.

Conrad TM, Lewis NE, Palsson BO. 2011. Microbial laboratory evolution in the era of genome-scale science. Mol Syst Biol. 7:509.

Conrad TM, et al. 2009. Whole-genome resequencing of Escherichia coli K-12 MG1655 undergoing short-term laboratory evolution in lactate minimal media reveals flexible selection of adaptive mutations. Genome Biol. 10:R118.

de Crécy-Lagard VA, Bellalou J, Mutzel R, Marlière P. 2001. Long term adaptation of a microbial population to a permanent metabolic constraint: overcoming thymineless death by experimental evolution of Escherichia coli. BMC Biotechnol. 1:10–16.

Desai MM, Fisher DS. 2007. Beneficial mutation-selection balance and the effect of linkage on positive selection. Genetics 176:1759–1798.

Desai MM, Fisher DS, Murray AW. 2007. The speed of evolution and maintenance of variation in asexual populations. Curr Biol. 17:385–394.

Doebeli M, Ispolatov I. 2010. Complexity and diversity. Science 328:494–497.

Friesen ML, Saxer G, Travisono M, Doebeli M. 2004. Experimental evidence for sympatric ecological diversification due to frequency-dependent competition in Escherichia coli. Evolution 58:245–260.

Gause GF. 1934. The struggle for existence. Baltimore (MD): Williams and Wilkins.

Gernst PJ, Lenski RE. 1998. The fate of competing beneficial mutations in an asexual population. Genetics 149:127–144.

Good BH, Rouzine IM, Balick DJ, Hallatschek O, Desai MM. 2012. Distribution of fixed beneficial mutations and the rate of adaptation in asexual populations. Proc Natl Acad Sci U S A. 109:4950–4955.
Gresham D, et al. 2008. The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. PLoS Genet. 4:e1000303.

Hardin G. 1960. The competitive exclusion principle. Science 131:1292–1297.

Herring CD, et al. 2006. Comparative genome sequencing of Escherichia coli allows observation of bacterial evolution on a laboratory timescale. Nat Genet. 38:1406–1412.

Hervas AB, Canosa I, Santero E. 2008. Transcriptome analysis of Pseudomonas putida in response to nitrogen availability. J Bacteriol. 190:416–420.

Hindré T, Knibbe C, Beslon G, Schneider D. 2012. New insights into bacterial adaptation through in vivo and in silico experimental evolution. Nat Rev Microbiol. 10:352–365.

Hughes JM, et al. 2012. The role of clonal interference in the evolutionary dynamics of plasmid-host adaptation. MBio 3(4):e00077–12.

Jackson RW, Johnson LJ, Clarke SR, Arnold DL. 2011. Bacterial pathogen evolution; breaking news. Trends Genet. 27:32–40.

Khan AI, Dinh DM, Schneider D, Lenski RE, Cooper TF. 2011. Negative epistasis between beneficial mutations in an evolving bacterial population. Science 332:1193–1196.

Langmead B, Trapnell C, Pop M, Salzberg SL. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 10:R25.

Levin BR. 1988. Frequency-dependent selection in bacterial populations (and discussion). Philos Trans R Soc Lond B Biol Sci. 319:459–472.

Li H, et al. 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25:2078–2079.

Maharjan R, Seeto S, Notley-McRobb L, Ferenci T. 2006. Clonal adaptive radiation in a constant environment. Science 313:514–517.

Maharjan RP, Seeto S, Ferenci T. 2007. Divergence and redundancy of transport and metabolic rate-yield strategies in a single Escherichia coli population. J Bacteriol. 189:2350–2358.

Maharjan RP, et al. 2012. The multiplicity of divergence mechanisms in a single evolving population. Genome Biol. 13:R41.

Milne I, et al. 2010. Tablet—next generation sequence assembly visualization. Bioinformatics 26:401–402.

Park SC, Krug J. 2007. Clonal interference in large populations. Proc Natl Acad Sci U S A. 104:18135–18140.

Rosenzweig RF, Sharp RR, Treves DS, Adams J. 1994. Microbial evolution in a simple unstructured environment: genetic differentiation in Escherichia coli. Genetics 137:903–917.

Tenaillon O, et al. 2012. The molecular diversity of adaptive convergence. Science 335:457–461.

Toft C, Andersson SG. 2010. Evolutionary microbial genomics; insights into bacterial host adaptation. Nature Rev Genet. 11:465–475.

Wang L, et al. 2010. Divergence involving global regulatory gene mutations in an Escherichia coli population evolving under phosphate limitation. Genome Biol Evol. 2:478–487.

Woods RJ, et al. 2011. Second-order selection for evolvability in a large Escherichia coli population. Science 331(6023):1433–1436.

Zerbino DR, Birney E. 2008. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 18:821–829.

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