The influence of cellular physiology on the initiation of mutational pathways in *Escherichia coli* populations

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The factors affecting the direction of evolutionary pathways and the reproducibility of adaptive responses were investigated under closely related but non-identical conditions. Replicate chemostat cultures of *Escherichia coli* were compared when adapting to partial or severe glucose limitation. Four independent populations used a reproducible sequence of early mutational changes under both conditions, with *rpoS* mutations always occurring first before *mgl*. However, there were interesting differences in the timing of mutational sweeps: *rpoS* mutations appeared in a clock-like fashion under both partial and severe glucose limitation, while *mgl* sweeps arose under both conditions but at different times. Interestingly, *malT* and *mlc* mutations appeared only under severe limitation. Even though the ancestors were genotypically identical, the semi-differentiated properties of bacteria growing with mild or severe glucose limitation sent the populations in characteristic directions. Mutation supply and the fitness contribution of mutations were estimated and demonstrated to be potential influences in the choice of particular adaptation pathways under severe and mild glucose limitation. Predicting all the mutations fixed in adapting populations is beyond our current understanding of evolutionary processes, but the interplay between ancestor physiology and the initiation of adaptation pathways is demonstrated and definable in bacterial populations.

Keywords: mutational sweeps; mutation rates; chemostat populations; glucose limitation; glucose transporters; hunger response

1. INTRODUCTION

Evolution is a biological process, yet we know little of the physiological influences on the progress of adaptation. Rules governing adaptive choices and mutation rates must exist, because evolution is quite reproducible in tightly confined situations. When replicate experimental populations of bacteria are continuously subjected to the same selection condition, the evolved characteristics generally result in similar changes of fitness and properties (Helling *et al.* 1987; Lenski & Travisano 1994). Evolution in experimental populations is also reported to show a clock-like increase in fitness over hundreds of generations, independent of the growth rate (Hartl & Dykhuizen 1979). Furthermore, at least four target loci altered in long-term parallel glucose-limited cultures are reproducibly the same (Manche *et al.* 1999; Notley-McRobb & Ferenci 1999a,b). Yet little is known about the cellular properties contributing to this reproducibility or how sensitive the outcome would be to minor environmental deviations. The choice of adaptation pathways in similar, but non-identical, environments is the subject of this communication.

To study the influences on the processes of evolution, we used a system capable of testing the reproducibility of adaptation. The approach involves chemostat cultures of bacteria maintained for hundreds of generations (Novick & Szilard 1951; Kubitschek 1970; Treves *et al.* 1998). Bacteria in chemostats are primarily selected for improved scavenging of the limiting nutrient (Dykhuizen & Hartl 1983; Helling *et al.* 1987). To test

whether adaptation followed similar paths with fixed but different levels of glucose limitation, mutations improving fitness under partial and severe glucose limitation were studied during 24 days of culture. Regulatory circuits prepare *Escherichia coli* for one of two distinct patterns of gene expression in nutrient-limited chemostat populations: nutrient scavenging at micromolar glucose concentrations and starvation survival when glucose levels drop below 10^{-7} M (Ferenci 1999, 2001). By starting genotypically identical bacteria on the same adaptive path, but with different patterns of gene expression in the ancestor, we can test how different levels of glucose limitation influence mutational adaptations. The glucose concentration was the only environmental difference between these conditions, but it forces the cells to grow at particular subsaturating growth rates. Partial and severe glucose limitation resulted in doubling times of *ca.* 1.1 h ($\mu = 0.6$ h⁻¹) and 7 h ($\mu = 0.1$ h⁻¹), respectively, and produced strikingly different adaptive outcomes.

To follow mutational processes, we used a 'toolbox' of genes affected by prolonged glucose limitation. Mutations in *rpoS* rapidly sweep glucose-limited cultures (Notley-McRobb *et al.* 2002a) and mutations in *mgl*, *malT* and *mlc* have also been demonstrated at a particular intermediate growth rate ($\mu = 0.3$ h⁻¹) (Notley-McRobb & Ferenci 1999a,b). Regulatory mutations in the *rpoS*, *mlc*, *malT* and *mgl* genes improved high-affinity glucose uptake through increased expression of *ompF*, *lamB*, *mglBAC* and *ptsG* (Notley-McRobb & Ferenci 1999a,b; Notley-McRobb *et al.* 2002a), which all influence fitness under glucose limitation. However, given the differences in regulation between severe and mild glucose limitation (Ferenci 2001), we suspected the fitness contribution of these

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mutations might be different at different growth rates. This notion could be tested, and we show that there is indeed a different selective advantage of mutations under closely related conditions.

A recognized factor in bacterial adaptation is the availability of mutations (de Visser *et al.* 1999). The spontaneous mutation rate in populations is not constant, and strong long-term selection for mutational adaptation selects for the enrichment of mutators in populations (Mao *et al.* 1997; Sniegowski *et al.* 1997; Taddei *et al.* 1997). Some chemostat populations under glucose limitation are also enriched for mutator mutations such as *mutS* or *mutY* (Notley-McRobb & Ferenci 2000; Notley-McRobb *et al.* 2002b). In this study, we wanted to exclude populations with these events, and to focus on the mutation rates as a function of growth rate in the absence of mutators. Hence, populations were screened for mutator isolates, and the mutation rates in the absence of mutators were measured under mild and severe glucose limitation. Recent studies of 'adaptive' mutations (Cairns *et al.* 1988; Foster 1999; McKenzie & Rosenberg 2001) consider mutation rates in starving bacteria in the non-uniform environment of colonies on agar plates; it was unclear whether mutation-rate changes could be observed in steady-state slowly growing bacteria. Indeed, mutation rates were strikingly different under mild and severe glucose limitation, extending observations that mutation rates were different under different types of nutrient limitation (Novick & Szilard 1951).

2. MATERIAL AND METHODS

(a) Bacterial strains

The bacterial strains used in this study are derivatives of *E. coli* K12. BW2952, an MC4100 derivative with a *malG-lacZ* transcriptional fusion (Notley & Ferenci 1995), was used as the starting strain in all chemostat experiments. The basal salts medium in all experiments was minimal medium A (MMA) (Miller 1972) supplemented with glucose, lactate or glycerol as specified for each experiment. Batch cultures contained 0.4% sugar and were harvested during mid-exponential growth. Tetrazolium and arabinose (TA) plates contained 5 g l⁻¹ of NaCl, 10 g l⁻¹ of tryptone and 1.5% agar.

(b) Culture conditions

Glucose-limited chemostats (80 ml) were set up as previously described (Death *et al.* 1993). The glucose concentration in the feed medium was 0.02%. The chemostats were maintained for the specified number of days at a dilution rate of 0.1, 0.3 or 0.6 h⁻¹. Populations were sampled daily by aseptic collection from the overflow port. Samples were subjected to the T5 resistance assay and testing for *rpoS* phenotype, and stored in 40% glycerol at -70 °C for later use. Samples were streaked on non-selective nutrient agar and randomly separated colonies were purified and tested in the phenotypic tests listed below.

The design of competition experiments in chemostats between *malT*-con mutants and wild-type bacteria, using *metC::Tn10* as the marker, was as previously described (Notley-McRobb & Ferenci 1999a).

(c) Mutations to T5 phage resistance in cultures

The frequency of T5-resistant mutants in a population was determined by the method of Helling *et al.* (1987). Samples from the chemostat population were mixed directly with a sus-

pension of bacteriophage T5 on TA plates as described previously (Notley-McRobb & Ferenci 2000).

(d) Mutator screening

A single colony of each of 20 random isolates at each time-point to be tested was inoculated into 4 ml L-broth, for screening of mutation rates. Mutation frequencies were tested on LB agar containing rifampicin at 100 µg ml⁻¹ as previously described (Notley-McRobb & Ferenci 2000).

(e) Phenotypic tests for *mgl*, *mlc*, *malT* and *rpoS* mutations

To detect *mgl*-con mutations, the initial rate of uptake of 1 µM of U[14C]-galactose by glycerol-grown chemostat isolates was determined using bacteria resuspended to an A₅₈₀ of 0.2 as described previously (Death & Ferenci 1993). For *mlc* changes, the increased sensitivities to the PtsG and PtsM substrates, methyl- α -glucoside and 2-deoxyglucose were tested as previously described (Notley-McRobb & Ferenci 2000). For *malT*-con mutants, β -galactosidase activity of the *malG-lacZ* fusion was measured on glycerol-grown isolates treated with chloroform and sodium dodecyl sulphate by the method of Miller (1972). For *rpoS*, the glycogen staining method for colonies from culture samples was used (Notley-McRobb *et al.* 2002a).

(f) Mutation analysis

Sequencing of the *mglD-galS* sequence and the *mgl* operator used the methods and primers previously described (Notley-McRobb & Ferenci 1999a). The sizes of large insertions were estimated by agarose gel electrophoresis after PCR using the same *mgl* primers.

3. RESULTS

(a) Course of appearance of four mutations under glucose limitation

Figure 1 illustrates the behaviour of four replicate cultures under each condition (dilution rate of $\mu = 0.1$ or 0.6 h⁻¹) that were sampled for 24 days. Daily, isolates from each culture were checked for the appearance of regulatory mutations in the *rpoS*, *mlc*, *malT* and *mgl* genes, which have been previously identified to improve high-affinity glucose uptake (Notley-McRobb & Ferenci 1999a,b; Notley-McRobb *et al.* 2002a). Interestingly, the tested mutations appeared in a reproducible sequence. In all cultures, the *rpoS* mutations appeared first and at similar time-points (figure 1a). Mutations in *mgl* followed, with the *mlc* and *malT* mutations occurring later and in a more scattered manner, at least in the slow-growing cultures. This result suggested that mutations in *malT* become beneficial only when other mutations are already present; this point is considered further below (§ 3c).

The timing of mutations in genes other than *rpoS* was dependent on the extent of glucose limitation. At the more extreme limitation, mutations in both *mgl* and *mlc/malT* appeared within the time scale of these experiments. In contrast, no *malT/mlc* mutants appeared in cultures with the faster growth rate. The difference is even more dramatic when one considers the number of generations covered in the experimental period. Mutations in *mgl* appeared within 14 generations at the slow growth rate in all four populations, but starting at 270–480 generations

Figure 1. Appearance of mutations as a function of specific growth rate in glucose-limited continuous culture. Eight independent populations were started with an *E. coli* K12 strain BW2952, an MC4100 derivative with a *malG-lacZ* transcriptional fusion (Notley & Ferenci 1995). The dilution rate was fixed at $D = 0.1 \text{ h}^{-1}$ in four populations and at 0.6 h^{-1} in the other four. The chemostats were maintained for 24 days and daily samples tested for mutations in *mgl*, *mlc*, *malT* and *rpoS* using phenotypic tests for each (Notley-McRobb & Ferenci 1999a,b; Notley-McRobb *et al.* 2002a). The appearance of each of the mutations is indicated in (a) for *rpoS* mutations; in (b) for *mgl* mutations; and in (c) for regulatory mutations in *mlc* (hatched boxes) and *malT* (solid boxes). The boxes indicate the day on which mutants first became more than 10% of the population; subsequent samples in each case contained increasing numbers of mutants with the corresponding mutations (results not shown).

at the faster growth rate. The *mlc* and *malT* changes appeared within 63 generations in the four populations under severe glucose limitation, but did not appear at all within 500 generations at the faster growth rate. Not only was there variation in the time of appearance of *malT* mutations under severe limitation, but also no *mlc* mutations were detected in two out of the four cultures. A possible explanation is that *mlc* mutations are redundant if *malT* mutations sweep first; the two loci both affect the

same regulatory target, namely *lamB*, with *malT* mutations having the stronger beneficial effect (Notley-McRobb & Ferenci 1999b). Overall, the data in figure 1 suggest that the selection pressures or mutational processes (or both) differ under mild and more extreme glucose limitation. The clock-like increase in fitness noted in glucose-limited cultures (Hartl & Dykhuizen 1979) is apparently not because of the same mutations at slow and fast growth rates; the

Figure 2. Mutation-rate differences for different growth rates. Three replicate chemostats were cultured at each growth rate ($D = 0.1 \text{ h}^{-1}$ (circles) and 0.6 h^{-1} (squares)). The cultures were tested for the appearance of neutral mutations in *fhuA*, which do not affect growth under glucose limitation. The mutations were detected by phenotypic testing for phage T5 resistance in culture samples as previously described (Notley-McRobb & Ferenci 2000). The data points for each of the three replicate cultures were plotted together to obtain the lines shown.

fitness increases at the faster growth rate must be the result of mutations not identified in this study.

(b) Mutation rates under mild and severe glucose limitation

The type and timing of mutations arising in different conditions could be influenced by the supply of mutations in distinct cultures or by the relative selective advantage of mutations. The rates of spontaneous mutation were compared by measuring the appearance of neutral mutations in replicate cultures growing under glucose limitation at dilution rates of 0.1 or 0.6 h^{-1} . Mutations in *fhuA* are neutral in the environments used and can be readily monitored through resistance to phage T5 (Kubitschek 1970). As shown in figure 2, the mutations resulting in T5 resistance in replicate populations appeared at a rate of 1.5×10^{-5} per generation at the slow growth rate and 5×10^{-7} per generation at the faster growth rate. Even taking into account the different generation times, mutation supply is still fivefold higher per day in samples nearer starvation. These results confirm and extend the results of earlier studies that demonstrated that growth limitation with different nutrients in chemostats resulted in distinct mutation rates (Novick & Szilard 1951).

The higher rate of T5-resistance mutations raised the possibility that mutational processes were distinct at low and high growth rates. A profile of mutational processes was obtained by sequencing mutations occurring in each of the populations. As shown in electronic Appendices A and B (available on The Royal Society's Publications Web site), the types of mutation present in *mgl* at slow and faster growth rates were characterized by DNA sequencing. At the 0.1 h^{-1} dilution rate, when *mgl* mutations appeared within the first few days (figure 1), the four populations contained a variety of mutations. Populations 3 and 4 contained a limited number of *mgl* changes, with

one allele dominant in each case. In contrast, populations 1 and 2 had a more varied assortment of mutations, including insertions and several types of point mutation. There was no indication that the elevated mutation rates in T5 resistance were associated with a particular DNA repair defect or an increased frequency of transpositional events.

In the four cultures growing at 0.6 h^{-1} , the *mgl* mutations occurred as later events. Yet the sequence changes associated with the faster-growing cultures were not appreciably different from those at 0.1 h^{-1} . Two of the faster populations were also dominated by individual *mgl* mutations, whereas the other two were more mixed. Population 10 was particularly polymorphic with regard to *mgl* mutations, and two insertion events as well as a frame shift and five different point mutations were detected. Overall, these results did not reveal consistent mutational-spectrum differences between slow- and fast-growing bacteria.

We can also discount an alternative strategy of overcoming mutation supply limitation through enrichment of mutator mutations (Mao *et al.* 1997; Taddei *et al.* 1997). No mutators were detected in screening the $D = 0.1 \text{ h}^{-1}$ cultures by published methods (Notley-McRobb & Ferenci 2000), but one (population 10) of the 0.6 h^{-1} cultures contained mutators. The mutators in population 10 were detectable (more than 10% of the population) from day 21 only, so the initial difference in mutation rates shown in figure 2 was still a physiological characteristic of bacteria resulting from nutrient limitation and/or growth rate. However, the great variety of *mgl* mutations in population 10 (see electronic Appendix B) may have been influenced by the presence of mutator.

(c) Selection pressures under mild and extreme glucose limitation

A possible explanation for the appearance or non-appearance of *malT* mutations in figure 1 was that the fitness advantage was different under the two selection conditions. Hungry and starving bacteria have distinct patterns of gene expression (Ferenci 2001) so it is possible that a particular level of expression may satisfy growth requirements under one condition more than the other. As shown in figure 3, competition experiments between wild-type and *malT*-con mutants indeed indicated that *mal* constitutivity was not always a selectively beneficial characteristic: it was beneficial at slow rates but negatively selected at faster growth rates.

Interestingly, the selective disadvantage of *malT* mutations appeared to change after 20–40 h, when *rpoS* mutations sweep the cultures. This was particularly striking at an intermediate growth rate ($\mu = 0.3 \text{ h}^{-1}$). Hence, it is likely that the fitness effect of *malT* mutations is a function of the evolving genetic background, consistent with the observation in figure 1 that *malT* mutations appear in populations only after *rpoS* and *mgl* sweeps. Nevertheless, the negative selection at 0.6 h^{-1} means a *mal* mutation is less likely to sweep the populations.

4. DISCUSSION

The reproducibility of mutational patterns in replicate populations, and the fixed order of appearance of ben-

Figure 3. The fitness contribution of a *malT*-con mutation at different growth rates. For growth competition experiments, the wild-type strain (BW2952) and the competing isolate (BW3453; isogenic to BW2952 except for a *malT*-con mutation) were genetically tagged by the insertion of a *metC::Tn10* marker from strain NK6027 (obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, CT) by transduction. The tagged wild-type and the isolate were grown independently in glucose-limited chemostats in the media as in § 2b except that 40 µg ml⁻¹ of methionine was added. After growth overnight at the given dilution rate in continuous culture, bacteria were mixed 50 : 50 to start competition at the same dilution rate. Bacteria in the mixed population were counted by serial dilution and plating on nutrient agar (for total counts) or nutrient agar containing 15 µg ml⁻¹ of tetracycline (for counts of the *Tn10*-tagged strain). To detect selective marker (*metC::tet*) effects, the reciprocal experiment was carried out with wild-type bacteria and *Tn10*-tagged isolate; no effect of the *Tn10* on competition was found (results not shown). Squares, 0.1 h⁻¹; circles, 0.3 h⁻¹; triangles, 0.6 h⁻¹.

eficial mutations, suggest that bacterial populations traverse adaptive landscapes in a fixed manner. Presumably, the equally reproducible deviation in outcomes between the two closely related but non-identical selection conditions is the result of the strong environmental constraints of whether bacteria are growing slowly or more rapidly under nutrient limitation.

Despite the same ancestor and selection for improved fitness under glucose limitation, two factors affected the outcome of mutational adaptation. First, severe glucose limitation led to 30-fold higher mutation rates, which offers a much greater repertoire for bacterial adaptation during slow growth or increased stress. The difference in mutation rates is nearly commensurate with that caused by *mutY* mutator mutations in faster-growing bacterial populations (Notley-McRobb *et al.* 2002b). It is interesting that mutators appeared in five out of 10 populations previously screened at $\mu = 0.3$ h⁻¹ (Notley-McRobb *et al.* 2002b), and one out of four at $\mu = 0.6$ h⁻¹. None were found in the four populations growing at $\mu = 0.1$ h⁻¹. The numbers of populations are too small to make general conclusions, but it is intriguing if slow growing populations generate sufficient beneficial mutations to avoid enrichment of mutators by secondary selection. It will be interesting to see in the future whether mutator enrichment is

more generally associated with adaptation of faster-growing bacteria.

Mutation supply is an obvious problem for small populations (de Visser *et al.* 1999) but rare mutations can change the path or rate of evolution even in the larger (1.6×10^{10} bacteria) populations studied here. For example, *malT*-con mutations are rare gain-of-function mutations requiring particular amino acid substitutions and so could be limited if mutation rates are low (Notley-McRobb & Ferenci 1999b). These results also extend the concept of 'adaptive' mutations found in the non-uniform environment of colonies on agar plates (Cairns *et al.* 1988; Foster 1999; McKenzie & Rosenberg 2001). However, the spectrum of mutations shown in electronic Appendices A and B does not readily distinguish pathways of mutagenesis in fast- and slow-growing bacteria.

The second major finding is that the selective advantage of mutations affecting *mal* genes is markedly different in more- and less-stressed cells. The *malT*-con mutation was beneficial at lower growth rates but surprisingly harmful to fitness under the lesser limitation. The advantage of *malT*-con under stress is understandable in that expression of *lamB*, which is important in glucose scavenging, is very depressed at $\mu = 0.1$ h⁻¹. At $\mu = 0.6$ h⁻¹, wild-type bacteria have far higher *lamB* expression (Notley & Ferenci 1995), which may be enough to satisfy the requirement for LamB protein under glucose limitation. This may lower the selective advantage of *malT* mutations, but does not explain the negative influence on fitness. We can only speculate that over-expression of *mal* genes may be harmful in ways not yet defined.

Although we cannot exclude other contributing factors, the differences in mutation rates and the selective advantage of mutations are in themselves sufficient to influence adaptation pathways and the temporal ordering of mutations. The physiological state of the ancestor cell influenced these two factors differently under severe and mild glucose limitation. Bacteria responded differently to distinct levels of stress because they were semi-differentiated physiologically. Bacteria respond to stress inputs in two ways. Specific to the particular stress, a physiological stress response is evoked (Storz & Hengge-Aronis 2000). In the current study, the separate hunger and starvation responses were the major contributors (Ferenci 2001) and the levels of stress used as the starting points for these experimental evolution studies elicit different patterns of gene expression (Ferenci 2001; Wick *et al.* 2001). The extent of the stress influenced not only the type of stress response but also the growth rate. A partial or major reduction in growth rate influences bacteria in many physiological ways (Ingraham *et al.* 1983). Both the stress input and the growth-rate input influence the physiological state of bacteria and, in turn, the mutation rate. A possible link between growth rate and mutation rate is through the signalling molecule ppGpp (Wright 1996). Our data suggest that both the supply of mutations and the selective usefulness of mutations affect the fixation of mutations, resulting in adaptation.

The major inference from these studies is that evolutionary pathways are strongly, but subtly, influenced by the physiological state of the ancestor cell. As shown for *malT* mutations, the benefit from a particular mutation is not identical at different levels of the same stress or with

different genetic backgrounds. These results are consistent with data obtained for antibiotic-resistance mutations involving multiple steps (Levin *et al.* 2000; Reynolds 2000). The selection advantage is always relative to a semi-differentiated state (as influenced by the environment and current genotype) and not a fixed level of fitness in the ancestor. The interplay between the environment and adaptive outcomes makes the prediction of evolution a highly complex undertaking unless one has detailed knowledge of the state of the cell that is about to obtain a beneficial mutation.

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