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SHORT REVIEW

The spread of a beneficial mutation in experimental bacterial populations: the influence of the environment and genotype on the fixation of *rpoS* mutations

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The spread of beneficial mutations through populations is at the core of evolutionary change. A long-standing hindrance to understanding mutational sweeps was that beneficial mutations have been slow to be identified, even in commonly studied experimental populations. The lack of information on what constitutes a beneficial mutation has led to many uncertainties about the frequency, fitness benefit and fixation of beneficial mutations. A more complete picture is currently emerging for a limited set of identified mutations in bacterial populations. In turn, this will allow quantitation of several features of mutational sweeps. Most importantly, the 'benefit' of beneficial mutations can now be explained in terms of physiological function and how variations in the environment change the selectability of mutations. Here, the sweep of *rpoS* mutations in *Escherichia coli*, in both experimental and natural populations, is described in detail. These studies reveal the subtleties of physiology and regulation that strongly influence the benefit of a mutation and explain differences in sweeps between strains and between various environments.
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Introduction

Spontaneous beneficial mutations contribute significantly to evolutionary change but remain one of the least understood aspects of biology (Orr, 1998; Hegreness *et al*., 2006). In the absence of fully characterized mutational sweeps, modelling mutational change was adopted in much of the early literature. Undoubtedly, the population biology approach and models of mutational takeovers were historically significant (Haldane, 1927; Fisher, 1930), but these and subsequent models necessarily needed to invoke assumptions on the fitness benefit conferred by mutations as well as their frequency in populations. The identity of beneficial mutations and the extent of the benefit have been elusive, although recent efforts have tried to estimate the distribution of benefits in experimental populations (Imhof and Schlotterer, 2001; Rozen *et al*., 2002; Barrett *et al*., 2006). This review focuses on a set of recently defined beneficial mutations arising in experimental continuous culture populations and what determines the conditional magnitude of their benefit under particular selection conditions.

With bacteria, beneficial sweeps should be readily studiable, but population changes called periodic selection events were first observed indirectly through monitoring of the fluctuations in the proportion of selectively neutral mutations (Atwood *et al*., 1951). Until recently, with the exception of a set of mutations limited to the *lac* system (see references in Watt and Dean, 2000), the nature of beneficial mutations was not easy to study. The few previously characterized beneficial mutations involved regulatory changes in expression (Novick and Horiuchi, 1961; Adams, 2004). The difficulties in identifying beneficial mutations were discussed in a perceptive review a decade ago (Lenski *et al*., 1998) and even in well-studied bacteria like *Escherichia coli*, there is considerable difficulty in predicting what kind of change constitutes a beneficial mutation in a novel environment. Nevertheless, there has been progress in the past 10 years in the analysis of two kinds of experimental evolution system with *E. coli* cultures. In the Lenski long-term sub-cultured populations, several types of DNA change and fitness contributions have been discussed (Cooper *et al*., 2001; Lenski *et al*., 2003; Schneider and Lenski, 2004; Crozat *et al*., 2005; Pelosi *et al*., 2006). There has also been progress in identifying mutations in chemostats (continuous cultures) evolving in the presence of limiting glucose (Treves *et al*., 1998; Manche *et al*., 1999; Notley-McRobb and Ferenci, 1999a, b, 2000; Notley-McRobb *et al*., 2002, 2003; Maharjan *et al*., 2006). Many of these examples deserve more detailed discussion, but in view of space limitations, this review is largely limited to one mutation that can be interpreted in terms of population sweeps, bacterial physiology and fitness effects under multiple growth conditions. This example illustrates

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many of the complex interplays between regulation, environment and mutation, which determine whether a mutation is beneficial. It also illustrates an example of trade-offs in evolution, long considered to be significant (MacArthur and Wilson, 1967), but for which detailed evidence has been scarce. In the case of *rpoS*, the conditionality of the beneficial and detrimental effects can also be discussed.

Strongly beneficial mutations in *rpoS* in *E. coli* populations growing under glucose limitation

The *rpoS* gene provides an excellent example of how counter-intuitive beneficial mutations can be. The σ^S protein encoded by *rpoS* is the second most important sigma factor in *E. coli* and responsible for the expression of around 10% of *E. coli* genes, mostly contributing to the general stress response (Weber *et al*., 2005). Because of its high position in the hierarchy of transcriptional regulators, σ^S was expected to be conserved in varying environments. Yet in studying the expression of *rpoS* in glucose-limited chemostats, it was serendipitously observed that *rpoS* mutations occurred, and indeed spread at rapid rates within a few generations of establishing glucose-limited chemostats (Notley-McRobb *et al*., 2002). As shown in Figure 1, the selection coefficient of *rpoS* mutants is the highest of the regulatory mutations already identified in glucose-limited populations (Notley-McRobb *et al*., 2003). These mutations in *rpoS*, *mlc*, *malT* and *mglD*/O genes, all increased fitness and transport of limiting nutrient by distinct regulatory mechanisms (Notley-McRobb and Ferenci, 1999a, b). The *mlc, mgl and malT* mutations resemble the earlier identified mutations and involve more limited regulatory changes in expression (Novick and Horiuchi, 1961).

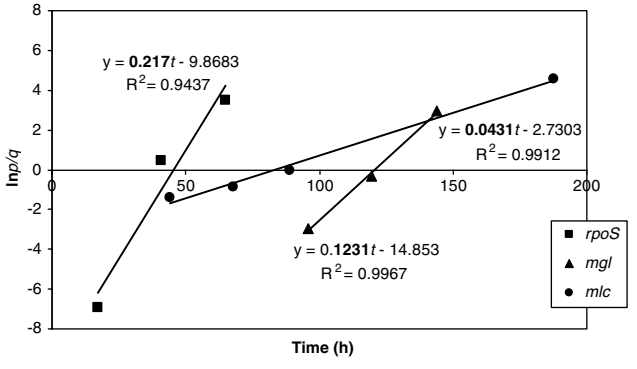

Figure 1 The selection coefficient of beneficial mutations spreading through *E. coli* populations. Bacteria inoculated into chemostats and subject to continuous glucose limitation accumulate mutations in several regulatory genes, including *rpoS, mglD* and *mlc* (Notley-McRobb *et al*., 2003). The spread of these mutations can be followed using simple phenotypic screens and the proportion of mutants relative to the rest of the population can be determined. With a dilution rate of $0.3\,h^{-1}$ and a population size of 2×10^{10} bacteria, the selection coefficient s is determined by the slope of the linear regression of $\ln[p(t)/q(t)]$, where $p(t)$ and $q(t)$ represent the relative frequencies of the strains at times t (Dykhuizen and Davies, 1980). The numbers in bold in the regression analyses indicate the selection coefficients *(s)* for each sweep. The timescale is not based on a single culture; the data were obtained from the populations and sweeps described in Notley-McRobb and Ferenci (2000, 2002).

Consistent with the high selection coefficient of *rpoS* sweeps, over 95% of the starting population is replaced in the cultures studied in Figure 1 within 3 days or 30 generations, growing at doubling times of 2.4 h. The takeover is even quicker, within 10–12 generations, at slower growth rates. Also interesting is that the sequence of sweeps in glucose-limited cultures is inversely related to the selection coefficients of the three mutations in Figure 1. The sweep order *rpoS* > *mgl* > *mlc* is reproducibly found in multiple replicate glucose-limited populations (Notley-McRobb *et al*., 2003). Whether selection coefficient always determine sweep order in evolving populations is unclear, but is clearly an important factor in large populations with no mutation supply problems. The decreasing selection coefficients in consecutive sweeps is also consistent with the finding that the fitness increase in evolving populations decreases asymptotically over extended periods of adaptation (Lenski *et al*., 1998).

Nature of *rpoS* mutations in glucose-limited populations

The majority of *rpoS* mutations accumulating in glucose-limited cultures are loss-of-function mutations with little or no residual RpoS protein. Sequencing of mutations from several populations has shown that sweeps such as in Figure 1 are due to concurrent enrichment of several distinct clones with distinct mutations (Notley-McRobb *et al*., 2002; unpublished data). The mutations include stop codons, deletions, insertions as well as point mutations. Given the population size of the chemostat cultures ($>10^{10}$ bacteria), it is to be expected that multiple spontaneous loss-of-function mutations are present and all would have a similar fitness benefit. Indeed, phenotypic screens for *rpoS* function in chemostat-derived mutants demonstrate similar, drastic effects.

The replacement of the parental population by several independently arisen *rpoS* mutants does have one important consequence. Superficially, the replacement of the chemostat population by the mutants is not diversifying in a phenotypic sense, but there are genetic implications. The separate genome lineages with different *rpoS* mutations established in this first sweep lead to the possibility of other mutations differentially hitchhiking with the selected *rpoS* mutations. This effect was most strikingly observed with mutator mutations that became prevalent in some chemostat populations by co-selection with a subset of the sweeping mutants (Notley-McRobb and Ferenci, 2000). Such events make seemingly straightforward periodic selections more complex and provide the source of later divergences in longer term populations. An example of this divergence was observed after 90 generations, when several *rpoS* alleles are stably established in chemostat populations, but with different mutational histories (Maharjan *et al*., 2006). The finding of divergence and the absence of purging periodic selections in experimental bacterial populations is not a new finding in itself and several explanations of diversification have been offered (Korona, 1996; Rainey and Travisano, 1998; Papadopoulos *et al*., 1999; MacLean and Bell, 2003). The *rpoS* findings add to explanations of diversity in populations.

The loss of σ^S is not a lethal defect in unstressed cells, so in normal buffered media at optimal temperature and pH, *rpoS* mutations do not cause a growth defect. However, when the culture medium is suboptimal or is subject to additional environmental stresses, the absence of stress resistance resulting from an *rpoS* null mutation becomes detrimental. Interestingly, the nature of the *rpoS* mutations in glucose-limited chemostats cultured at acidic pH (5.5) is altered, with a predominance of partial or attenuated mutations (Notley-McRobb *et al*., 2002). Such attenuated *rpoS* mutations, also observed in long-term stationary phase cultures (Zambrano *et al*., 1993), permit a reduced level of stress resistance but greater fitness in chemostats or stationary phase. The interesting antagonistic trade-offs that determine the benefit of *rpoS* mutations is considered below.

The selectability of *rpoS* mutations as a function of the environment and strain genotype

The selection pressures on *rpoS* are largely due to the competition between the sigma factors RpoS (σ^S) and RpoD (σ^D) for a limiting number of RNA polymerase core subunits (Zhou and Gross, 1992; Farewell *et al*., 1998; Jishage and Ishihama, 1999). RpoS switches the cell to stress resistance whereas RpoD is needed for vegetative growth; an imbalance between the two can reduce the fitness of bacteria in particular situations. The hypothesis used to explain *E. coli rpoS* mutations in stationary phase batch cultures (Zambrano *et al*., 1993) or in steady-state glucose-limited chemostat populations (Notley-McRobb *et al*., 2002) is that mutations alleviate sigma factor competition and improve nutrient scavenging by increasing expression of RpoD-dependent genes (Ferenci, 2003). The trade-off in losing σ^S function is that *rpoS* mutants exhibit reduced resistance to stresses such as prolonged starvation, high pressure, high osmolarity, low pH as well as survival in the gastrointestinal tract (Cheville *et al*., 1996; Waterman and Small, 1996; Price *et al*., 2000; Dodd and Aldsworth, 2002; Bhagwat *et al*., 2006).

The antagonistic pleiotropy imposed by the sigma factor competition is one of several examples of the balancing of self-preservation and nutritional competence, or SPANC balancing, in bacteria (Ferenci, 2005). *rpoS* mutations, being beneficial in some settings but not others, exhibit complex environment-by-genotype interactions that are amenable to study. The selection pressures on *rpoS* are easiest to explain in schematic form as shown in Figure 2. Despite the overall complexity of the RpoS system, the rate at which *rpoS* mutations sweep populations in a particular environment can be reduced to a few quantifiable components. The first of these is the negative fitness effect on nutrient uptake, nutritional competence and vegetative growth resulting from high RpoS levels (Figure 2a). A second fitness input is the positive fitness contribution due to RpoS involvement in stress resistance in a particular environment. As found recently (King *et al*., 2006), the magnitude of both is sensitive to the particular surroundings. For example, at high osmolarity as shown as the stress in Figure 2a, the resistance due to RpoS is most important, and balances the nutritional advantage of elevated transport even under glucose limitation. In other more healthy environments, such as nutrient limitation at neutral pH (Figure 2a), the cost of RpoS-limiting transport outweighs the stress resistance benefit and *rpoS* mutations readily sweep populations. The type of *rpoS* allele enriched will also influence the magnitude of the costs and benefits in a sweep. When partial loss of function occurs, in some environments (Zambrano *et al*., 1993; Notley-McRobb *et al*., 2002), the fitness changes are less than with null mutants. The rate of *rpoS* mutation accumulation and type of mutation, as a function of the environment, have been estimated (King *et al*., 2006) and are discussed in the next section.

Environmental determinants in the spread of *rpoS* mutations

The contribution of selection pressures to the magnitude of a sweep was measured in 15 controlled environments, including the eight shown in Figure 3 (King *et al*., 2006). We measured the rate of displacement of wild-type bacteria by *rpoS* mutants under nutrient limitation with and without additional stresses. As shown in Figure 3, there was considerable variation in the rate of sweeps. As described by the scheme in Figure 2, *rpoS* takeovers should occur particularly when RpoS has a strongly negative effect on fitness, that is when σ^S levels are high but when a stress response is not needed for viability. In Figure 2a, this combination of events is shown for nutrient-limited bacteria in an otherwise unstressed environment.

The estimation of the negative effect was carried out with bacteria in a chemostat culture by measuring the transport of limiting nutrient. In chemostats, transport rate at limiting substrate concentrations is related to fitness (Novick and Szilard, 1950; Hansen and Hubbell, 1980; Notley-McRobb and Ferenci, 1999a). The *rpoS* influence is measurable from a comparison of glucose uptake rates in bacteria with and without intact *rpoS*, comparing isogenic strains differing only in *rpoS*. The inactivation of RpoS and removal of sigma factor competition indeed increase expression of all genes involved in glucose uptake, which are RpoD-dependent (Notley-McRobb *et al*., 2002; Seeto *et al*., 2004). By measuring glucose transport in each of the modified environments in *rpoS* and *rpoS*$^+$ bacteria, the repressive effect can be revealed and compared to the magnitude of the selection pressure for loss of *rpoS* in experimental sweeps.

As shown in Figure 3, environments such as anaerobiosis prevent or greatly reduce the spread of *rpoS* mutations when compared to aerobic glucose-limited chemostats at identical growth rates. At the same time, the environments still provide a selection pressure for the loss of RpoS, since a transport difference between *rpoS* and *rpoS*$^+$ bacteria was measurable in all cases. The observation that *rpoS* mutants did not spread in proportion to the transport difference is consistent with the prediction in Figure 2 that a fitness effect provided by stress resistance also contributes to the magnitude of the *rpoS* sweep. A reservation at this point is that we have no evidence that the transport difference is linearly related to fitness and this needs detailed analysis in the future. Nevertheless, in the case of anaerobiosis, low tempera-

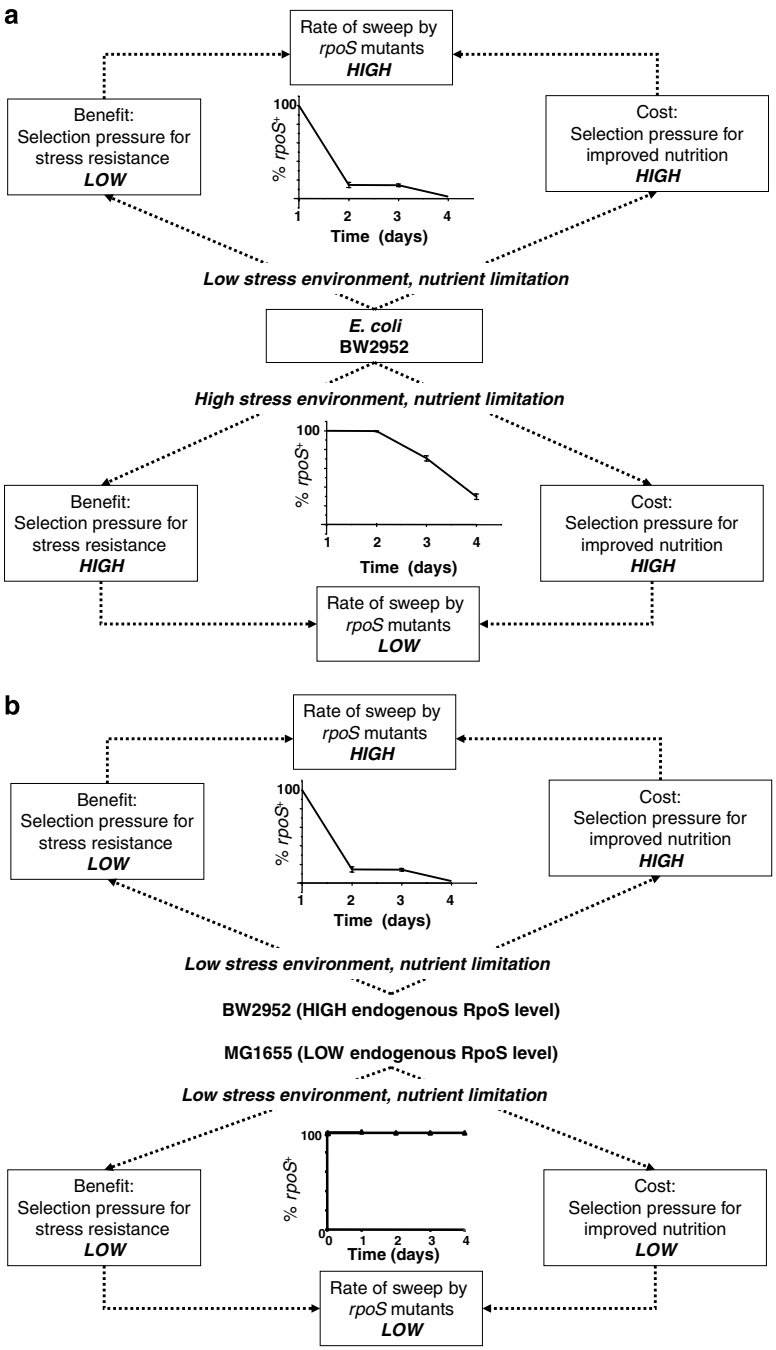

Figure 2 Major influences affecting the selectability of *rpoS* mutations in *E. coli*. The spread of *rpoS* mutations in *E. coli* populations is affected by the environment (**a**) and the genotype of the strain (**b**). (**a**) The environment determines the level of cellular σ^S through multiple input signals (Weber *et al*., 2005), and is higher under stress conditions. A particular concentration of σ^S is postulated to have two effects on fitness. First, σ^S contributes to viability by inducing the general stress response and provides a fitness contribution that is zero in unstressed bacteria (e.g. at 37 °C, pH 7, no osmotic stress, no oxidative stress, etc.) but positive under suboptimal conditions (e.g. at 25 °C, pH 5.5, high osmolarity etc.). In (**a**) the high-stress environment inset is with high osmolarity (King *et al*., 2006). In unstressed bacteria, a high level of σ^S has a negative effect on fitness because it competes with σ^D for available RNA polymerase and reduces expression of vegetative genes dependent on σ^D. The competition results in lower expression of functions, like transport, essential for growth. The transport difference between *rpoS*$^+$ and *rpoS*$^-$ bacteria in a particular environment is indicative of this negative fitness effect (Figure 3). Especially with low-nutrient environments (either limitation for good substrates like glucose or maltose, or growth with poor carbon sources like acetate that give low growth rates), *rpoS* expression is elevated in otherwise unstressed bacteria. The negative transport effect is high under these conditions (see Figure 3 below), so results in high rates of *rpoS* sweep and provides a selection pressure for the *rpoS* mutation (see insets for the pattern of *rpoS* sweeps in each condition; the data on glucose-limited chemostats with and without osmotic stress is from King *et al*., 2006). (**b**) Different strains of *E. coli* (both lab strains and natural isolates) vary in their σ^S content (King *et al*., 2004; Bhagwat *et al*., 2006). The genotype of *E. coli* determines σ^S levels, as the concentration of σ^S under stress conditions varies considerably between strains (Ferenci, 2005). Strains with genotype-determined high levels of RpoS protein (such as BW2952) have a greater cost (in terms of transport effect), even more under selection for *rpoS* mutations. MG1655 on the other hand has lower endogenous σ^S and the selection pressure for rpoS mutation is reduced. The inset *rpoS* sweep data is from King *et al*. (2004).

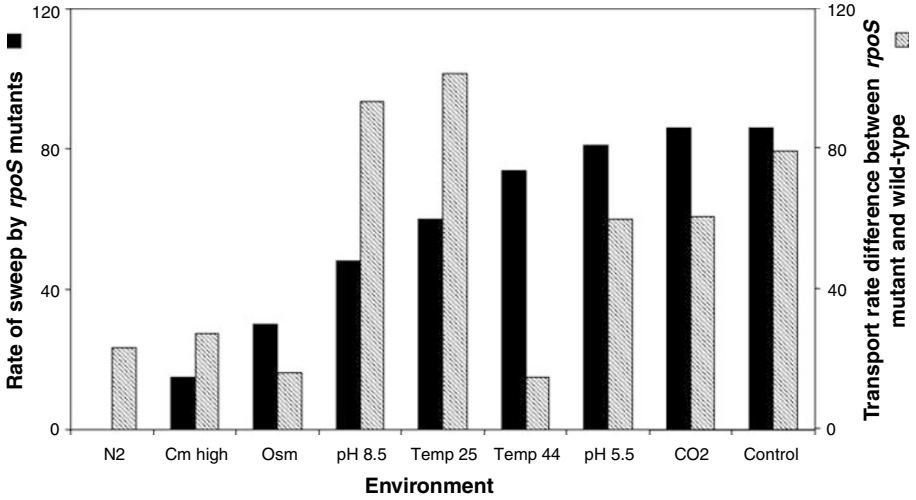

Figure 3 Estimation of the rate of sweeps and the selection pressure for beneficial mutations in *rpoS*. The 'control' in Figure 3 represents the cultures of *E. coli* grown in glucose-limited chemostats at 37 °C, pH 7, no osmotic stress, with aeration and at $D = 0.1\,h^{-1}$. This environment was modified by changing the medium or atmosphere in eight different ways indicated in Figure 3 and described in more detail in King *et al.* (2006). Briefly, CO_2 represents a culture atmosphere in which N_2 was replaced by CO_2; Temp or pH indicates a changed culture temperature or pH respectively; Osm indicates elevated osmolarity of the medium; Cm high indicates growth in the presence of sublethal chloramphenicol concentrations; N_2 represents an anaerobic environment. In each environment, the magnitude of the sweep was obtained from measuring the increasing proportion of *rpoS* bacteria over the first 4 days of culture. A high value is indicative of rapid sweeps. The transport rate difference between *rpoS*$^+$ and *rpoS*$^-$ bacteria was determined directly under each growth condition as also described in King *et al.* (2006).

ture or high pH, the selection of *rpoS* mutants was much lower than could be explained by the considerable magnitude of the transport cost. Other findings are also consistent with the significance of σ^S in particular environments such as under anaerobiosis (King and Ferenci, 2005). Although additional factors other than those considered in Figure 2 may contribute, the simplified scheme in Figure 2 will provide a means of analysing, for the first time, the physiological factors influencing a mutational sweep.

The study of *rpoS* sweeps illustrates the exquisite sensitivity of the fitness benefit to environmental conditions. The conditionality of fitness effects is even more dramatically demonstrated by *malT* mutations in chemostat cultures. These mutations switch from being beneficial to detrimental simply by changing the growth rate. Under glucose limitation, *malT* mutations are beneficial and selected at $D = 0.1\,h^{-1}$, near-neutral at $0.3\,h^{-1}$ but are detrimental at $0.6\,h^{-1}$ (Notley-McRobb *et al.*, 2003). Selectability of mutations is that sensitive to changes in bacterial physiology.

Strain polymorphisms and the influence of genotype on the spread of *rpoS* mutations

One of the several unexpected findings on the spread of *rpoS* mutations was that the speed and extent of the sweep shown in Figure 1 was remarkably strain-specific in different *E. coli* strains. As shown in Figure 2b, the benefit conferred by *rpoS* mutations under identical conditions of glucose limitation showed considerable strain variation. In some strains, the *rpoS* fitness benefit in chemostats is even greater than with BW2952, the strain generally used in my lab as the ancestor in evolution experiments. In other strains such as the often-used MG1655, the *rpoS* mutations are undetectable in glucose-limited cultures at times when BW2952 populations are already $>95\%$ *rpoS* (King *et al.*, 2004 and Figure 2b). Consequently, the host genotype is as important as the environment in determining the benefit of the *rpoS* mutation.

The underlying difference between strains is in the control of the endogenous level of σ^S protein in the cell. Under identical growth conditions, strains vary as much as 10-fold in σ^S protein per cell (King *et al.*, 2004). High endogenous RpoS results in higher sigma factor competition against RpoD, and *E. coli* strains with high σ^S levels indeed accumulated *rpoS* mutations at a higher rate during growth under nutrient limitation, relieving the competition. The high-σ^S strains have a reduced fitness in any environment where nutrition is the selective determinant. Disruption of *rpoS* indeed improves nutritional capability not just under glucose limitation but also with many poor carbon sources of *E. coli* like acetate or succinate (Chen *et al.*, 2004; King *et al.*, 2004).

The genetic basis underlying differences in σ^S levels is unknown, although intragenic polymorphisms undoubtedly contribute (Atlung *et al.*, 2002). Indeed, the widespread occurrence of polymorphisms in and near *rpoS* indicates that the selection pressures on the SPANC balance described in Figure 2 occur in natural environments (Ferenci, 2003). Consequently, changes in the SPANC trade-off in nature results in heterogeneity in several phenotypes associated with stress resistance (Bhagwat *et al.*, 2006). Many inputs control σ^S levels at the transcriptional and post-transcriptional phases of *rpoS* expression, so changes in numerous genes could influence protein levels and stress resistance. One recently identified contributor is linked to the *spoT* region present in a strain (Spira and Ferenci, 2008). *spoT*, also involved in global gene regulation, may also be expected to be subject to the same SPANC trade-offs as *rpoS* (Spira and Ferenci, 2008). No doubt other extragenic polymorphisms also explain σ^S level heterogeneity.

The fixation of *rpoS* mutations

Despite the magnitude of the fitness advantage conferred by *rpoS* mutations in high-RpoS strains under glucose limitation, none of the eight populations studied so far became 100% *rpoS*. The proportion of *rpoS*$^+$ bacteria decreased to below 1% in some populations, but the *rpoS*$^+$ sub-population was stably maintained. Indeed, in all chemostat cultures studied, the proportion of *rpoS*$^+$ bacteria recovered within 100 generations to become >30% of the population (Maharjan *et al*., 2006). An important question arising from these findings is if such very strongly beneficial mutations as in *rpoS* do not lead to complete fixation, is it likely that any mutation in a large bacterial population purges diversity? The ever-increasing complexity of long-term populations almost to the level of individuality (Papadopoulos *et al*., 1999; Maharjan *et al*., 2007) suggests a negative answer to this question. The universality of this conclusion will of course depend on data with other cell types and experimental systems.

The co-existence of multiple *rpoS* genotypes and the co-existence of both *rpoS*$^+$ and *rpoS* sub-populations are the likely sources of divergence in chemostat populations (Maharjan *et al*., 2006). More weakly beneficial mutations than those in Figure 1 have almost no chance of leading to fixation, and the slow turnover of sub-populations over several weeks of further adaptation in chemostats suggests that purging periodic events are not a feature of continually adapting bacteria. Beyond the first 50 generations under glucose limitation, the mutations with high selection coefficients are supplemented by numerous other mutations affecting multiple phenotypic characteristics such as regulation, metabolism, transport and outer membrane permeability (Maharjan *et al*., 2006). Because of space considerations, the diversifying effects of mutations with low selection coefficients will not be described here. Nevertheless, the incomplete fixation of mutations and the co-existence of mutations was a previously underestimated aspect of bacterial population change, but is also being observed in populations other than chemostat cultures (de Visser and Rozen, 2006). A possible basis of diversification is frequency-dependent selection (Friesen *et al*., 2004) and this is the explanation used to explain diversification in a more complex mixture of resources (Barrett and Bell, 2006). In our chemostat isolates, however, frequency-dependent selection was tested but does not explain the diversity in evolving chemostat populations (Maharjan *et al*., 2006, 2007).

Conclusions and prospects

The 'benefit' in beneficial mutations is a function of the interplay between genotype, environment and the physiology of a cell. In the case of *rpoS* mutations, the selectivity and speed of mutational spread is determined by the quantifiable trade-off costs in a particular environmental setting. The sensitivity of the benefit, the incomplete fixation of strongly beneficial mutations and the demonstrated divergence associated with weakly beneficial mutations are inconsistent with simple models of bacterial evolution. A simple black-box approach to bacterial properties in population models is no longer justifiable.

Up to now, the *rpoS* sweep represents one of the few beneficial mutations that have been amenable to detailed analysis. In the future, the identification and investigation of beneficial mutations will be easier and facilitated by the application of genomics. In the past few months, microbial genomes of lab-evolved organisms have been sequenced and mutations identified (Herring *et al*., 2006; Velicer *et al*., 2006). The complete cataloguing of all the mutations removes the earlier restrictions of trying to guess what beneficial mutations arise as well as the constraint of our incomplete understanding of organisms.

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