Heredity (2013) 110, 10–18

www.nature.com/hdy

Heredity

ORIGINAL ARTICLE

The basis of antagonistic pleiotropy in *hfq* mutations that have opposite effects on fitness at slow and fast growth rates

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Mutations beneficial in one environment may cause costs in different environments, resulting in antagonistic pleiotropy. Here, we describe a novel form of antagonistic pleiotropy that operates even within the same environment, where benefits and deleterious effects exhibit themselves at different growth rates. The fitness of *hfq* mutations in *Escherichia coli* affecting the RNA chaperone involved in small-RNA regulation is remarkably sensitive to growth rate. *E. coli* populations evolving in chemostats under nutrient limitation acquired beneficial mutations in *hfq* during slow growth ($0.1\,h^{-1}$) but not in populations growing sixfold faster. Four identified *hfq* alleles from parallel populations were beneficial at $0.1\,h^{-1}$ and deleterious at $0.6\,h^{-1}$. The *hfq* mutations were beneficial, deleterious or neutral at an intermediate growth rate ($0.5\,h^{-1}$) and one changed from beneficial to deleterious within a 36 min difference in doubling time. The benefit of *hfq* mutations was due to the greater transport of limiting nutrient, which diminished at higher growth rates. The deleterious effects of *hfq* mutations at $0.6\,h^{-1}$ were less clear, with decreased viability a contributing factor. The results demonstrate distinct pleiotropy characteristics in the alleles of the same gene, probably because the altered residues in Hfq affected the regulation of expression of different genes in distinct ways. In addition, these results point to a source of variation in experimental measurement of the selective advantage of a mutation; estimates of fitness need to consider variation in growth rate impacting on the magnitude of the benefit of mutations and on their fitness distributions.
Heredity (2013) **110,** 10–18; doi:10.1038/hdy.2012.46; published online 21 November 2012

Keywords: *Escherichia coli*; experimental evolution; fitness variance; growth rate; continuous culture; regulatory mutations

INTRODUCTION

Fitness increases due to mutations are vital for evolutionary success. The selective advantage of a mutation, *s*, is important because it affects the probability of its fixation in populations (Haldane, 1927). The precise benefit of a beneficial mutation is, however, complicated by several factors, including interactions between mutations (epistasis (Chou *et al.*, 2011; Khan *et al.*, 2011; Ostman *et al.*, 2012)). Mutations in genes that affect more than one trait (pleiotropy (Stearns, 2010)) are also complicated, especially if the pleiotropic mutation negatively affects other cellular properties (antagonistic pleiotropy). With regulatory mutations that exhibit epistasis, pleiotropy or both, the magnitude of *s* may be subject to factors that lead to its variance in conditions quite close to the selection environment. The range of environments over which *s* is beneficial is, in general, poorly defined in experimental studies. For mutations exhibiting antagonistic pleiotropy, the question of where in an environmental landscape a mutation switches from beneficial to deleterious is a fundamental one. It is this question we address in the current communication.

Numerous examples of antagonistic pleiotropy have been previously found in laboratory evolution experiments with bacteria, in which fitness gains in the selection environment are due to mutations that affect fitness elsewhere (Cooper and Lenski, 2000; Cooper *et al.*, 2001; MacLean *et al.*, 2004; Ferenci, 2005; Ostrowski *et al.*, 2008). According to Cooper and Lenski (2000), two properties associated with antagonistic pleiotropy are that replicate evolving lines should exhibit parallel decay of functions (those that trade-off with fitness in the selection environment), and that most losses should occur early (when adaptation is fastest). The inverse need not hold, that is, parallel pleiotropic changes are not a necessary consequence of adaptive mutations having antagonistic pleiotropy. Here, we discuss several independently arising *hfq* mutations in *E. coli* evolving in glucose-limited chemostat cultures that match the Cooper/Lenski predictions and show antagonistic pleiotropy. In contrast to the previously described examples cited above, a novel aspect is that the new *hfq* mutations switch from beneficial to deleterious even in the same medium environment, but at different growth rates. This unexpected finding prompted us to investigate the mechanism of antagonistic pleiotropy in *hfq* mutations and also to consider more widely the consequences of growth rate as a factor in *s* determination.

There is a fundamental reason for considering the relationship of *s* to growth rate. In an evolving population or in competition experiments, sweeps occur because beneficial or deleterious mutations cause different growth rates in a mutant (μ_m) to that found with ancestral bacteria in the same environment (μ_w). It matters a great deal how different μ_m and μ_w are because physiological studies have demonstrated that growth rate strongly affect patterns of gene expression, ribosome synthesis and metabolic pathways (Tweeddale *et al.*, 1998; Wilson and Nierhaus, 2007; Klumpp *et al.*, 2009; Scott

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Received 19 February 2012; revised 10 April 2012; accepted 13 April 2012; published online 21 November 2012

et al., 2010). Stress responses are increasingly induced at slower growth rates, diverting resources from vegetative fitness functions (Ferenci, 2005, 2007). Hence, a consequence of differences between μ_m and μ_w in an evolving population is that an ancestor and mutant inevitably differ in molecular composition in the same environment, not just as a result of a mutation, but also as a consequence of growth rate-determined patterns of gene expression. So how much impact does growth rate context caused by μ_m have on the estimation of *s* as a measure of fitness?

Growth-rate affected context can potentially have an impact on fitness and epistatic interactions, especially with the many pleiotropic regulatory mutations important in evolution (Barrier et al., 2001; Philippe *et al.*, 2007). Changes in intracellular context may have profound implications, particularly with regulatory mutations involved in a web of epistatic interactions, (Le Gac and Doebeli, 2010). Evidence for context influence additionally comes from experiments in which applied stresses change the fitness effects of particular mutations (Kishony and Leibler, 2003) and the strong genotype-by-environment interactions in the effects of a global regulatory mutation (King *et al.*, 2006). In most cases, how a mutational benefit interacts with a changing cellular context is not well understood, but a recent study showed fitness of a metal uptake mutation increased with growth rate (Chou *et al.*, 2009).

The variance and magnitude of *s* are of intense interest in population biology (Orr, 2009), but the interplay between fitness and growth is rarely considered in the experimental assessment of fitness (Chevin, 2011). There is no consistent method of standardizing growth rates during fitness measurements in competition assays and in some studies, fitness is actually measured as a difference in growth rates (MacLean and Buckling, 2009; Lind *et al.*, 2010). In others, *s* is measured over periods (overnight culture or 24 h) in which growth rate is non-constant (for example, (Elena and Lenski, 2003; Perfeito *et al.*, 2007; McDonald *et al.*, 2011)). Here, we directly investigated the fitness of *hfq* mutants at growth rates in continuous cultures fixed by the chemostat culture method. In a glucose-limited chemostat, bacteria reduce the concentration of limiting nutrient to a level that maintains a constant, suboptimal growth rate. By setting the dilution rate of fresh medium into a chemostat, a range of growth rates can be established. Bacterial growth rate is a saturable function of nutrient level, so residual glucose levels increase with increasing dilution rate. With this system, one can ask not only whether the same mutations are selected at low/high dilution rates (that is, low/high residual resource levels), but also further investigate whether mutations beneficial at one dilution rate are also beneficial at the other dilution rates. Previous studies have shown that some mutations are indeed selected at both $D = 0.6\,h^{-1}$ and $D = 0.1\,h^{-1}$ cultures, but some are not (Notley-McRobb *et al.*, 2003). So here we measured the fitness of pleiotropic mutations under controlled chemostat conditions over a sixfold range of dilution rates ($D = 0.1$–$0.6\,h^{-1}$).

The pleiotropy of *hfq* mutations is mechanistically interesting because Hfq is at the center of a wide network of bacterial environmental responses controlled through dozens of small RNAs, Hfq being an RNA chaperone (Tsui *et al.*, 1994; Majdalani *et al.*, 2005). Mutations in *hfq* cause multiple effects (such as decreased growth yields, altered supercoiling of plasmids in stationary phase, increased cell size, osmosensitivity and increased sensitivity to ultraviolet light (Tsui *et al.*, 1994)), and confer a selective advantage in slow-growing bacteria limited by several nutrients, including glucose, inorganic phosphate and lactate (Conrad *et al.*, 2009; Maharjan *et al.*, 2010; Wang *et al.*, 2010). Evidence described here suggested *hfq* mutations were less common in fast-growing $D = 0.6\,h^{-1}$ cultures. The multiplicity of roles of Hfq in imparting a benefit under glucose limitation (Maharjan *et al.*, 2010) also suggested its benefit is sensitive to physiological context. Here, we describe the plasticity of the benefit of several *hfq* mutations as a function of growth rate in an otherwise constant environment and define how it changes from a beneficial to a deleterious mutation.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacteria were cultured in L-broth (Miller, 1972) or minimal medium A (MMA) supplemented with either 0.2% (w/v) glucose for batch cultures or 0.02% (w/v) glucose in chemostats. Media were also supplemented with 1 μg ml^{-1} thiamine and 1 mM $MgSO_4$. For chemostat competition experiments 4 μg ml^{-1} methionine was also added to the medium. Conditions for long-term chemostats are described in Maharjan *et al.* (2006). All growth was carried out at 37 °C and the growth rate was controlled by setting the dilution rates of medium supply to the culture vessel according to the standard chemostat practice (Ferenci, 2007).

Strains used in this study are listed in Table 1. Mutant *hfq* alleles were transferred into ancestral background from the evolved isolates by P1 transduction (Miller, 1972). First, a *purA*::*tet* cassette was introduced into the ancestral strain using the protocol described in Yu and Court (1998). The proximity of *purA* to *hfq* allowed for ~90% co-transduction, allowing for selection of *purA*$^+$ transductants followed by confirmation of successful *hfq* transfer by sequencing.

Maximum specific growth rate measurement

To measure the maximum specific growth rate (μ_{max}), overnight culture of wild-type and *hfq* mutants grown in 5 ml MMA supplemented with 0.2% glucose in McCartney bottles were diluted 100-fold in 200 μl of the same medium in 96-deep-well plates (clear, polystyrene, flat-bottom from BD, Sydney, NSW, Australia). The plates were incubated in micro-plate reader (BMG Labtech 96, Ortenberg, Germany) at 37 °C with shaking at 300 r.p.m (double orbital mode). Growth of cultures were monitored by measuring the optical density of cultures at 600 nm at 15 min intervals and processed using the BMG Labtech Omega MARS data analysis software (Melbourne, VIC, Australia). The μ_{max} of each culture were then calculated from the linear regression of the exponential growth phase after plotting the culture optical density$_{600}$ in logarithmic scale against the time.

Sensitivity to α-methyl glucoside

Sensitivity to α-methyl glucoside (α-MG) was determined by growth on minimal agar plates containing 0.2% (w/v) glycerol and α-MG at a concentration of 0.5% (w/v). Strains were cultured overnight in MMA

Table 1 Bacterial strains used in this study

Strain	*Relevant genotype*	*Reference*
BW2952	F- araD139 D(argF-lac)U169 rspL150 deoCl *relA*1 thiA ptsF25 flb5301 rbsR *malG*::placMu55 (*malG*::*lacZ*)	Ferenci *et al.* (2009)
BW4001	Chemostat evolved isolate	Maharjan *et al.* (2010)
3 × 17	Chemostat evolved isolate	This study
3 × 27	Chemostat evolved isolate	This study
BW5155	BW2952 *purA*::*tet*	Wang *et al.* (2010)
BW5035	BW2952 *hfq*::*amp*	This study
BW5163	BW2952 *hfq*Y25D	This study
BW5164	BW2952 *hfq*L46W	This study
BW5165	BW2952 *hfq*IS1	This study
BW6034	BW2952 *hfq* S60L	This study
BW3454	BW2952 *metC*::Tn*10*	Notley-McRobb and Ferenci, (1999)

supplemented with 0.2% glycerol and 2 µl of these cultures were spotted onto α-MG plates. Plates were incubated overnight at 37 °C and successful growth indicated resistance to α-MG (Wang *et al.*, 2010).

Detection of RpoS status

The level of RpoS was estimated by staining with iodine solution; the intensity of the brown color varies according to the glycogen level in the cell, which in turn is controlled by the RpoS level in the cell (Notley-McRobb *et al.*, 2002).

β-galactosidase assay

β-galactosidase activity of the *malG-lacZ* fusion present in ancestor and all used strains (Notley and Ferenci, 1995) was determined as described in Miller (1972). For initial characterization, cells were grown in MMA supplemented with 0.2% (w/v) glycerol overnight and then subcultured in the same medium until optical density at 600 nm was ~0.28–0.7. For characterization of phenotypes in chemostats at different dilution rates, cells were grown in chemostats at the relevant dilution rate for 40 h.

Outer membrane protein analysis

The outer membrane protein was prepared by disrupting cells in a French pressure cell and separated by SDS-polyacrylamide gel electrophoresis as described previously (Maharjan 2006). Briefly, equal amount of cells (1.6×10^{10} cells) from 24-hour old 80-ml chemostat cultures were harvested by centrifugation, and the pellet was washed twice with 10 ml of 10 mM HEPES buffer (pH 7.4), resuspended in 10 ml of the same buffer. The cell suspension was then broken by passage three times through a French pressure cell at 6500 lb in^{-2}. The disrupted membrane was pelleted by centrifugation at 35 000 *g* for 1 h at 4 ºC. The membrane pellet was resuspended in 200 µl of sample buffer (50 mM Tris–HCl, 4% SDS, 16% glycerol, 0.0048% bromophenol blue, 4.8% β-mercaptoethanol).

The outer membrane samples were boiled for 5 min at 100 °C and centrifuged for 10 min at 14 000 r.p.m in an eppendorf microcentrifuge (model 5412). The proteins samples from equal numbers of cells (10 µl) were then separated on a 12% acrylamide gel in the presence of 8 M urea by electrophoresis, and protein bands were stained with 0.04% Coomassie blue R and destained with 10% acetic acid.

PCR protocols and mutation analysis

PCR amplification of a 697 bp region flanking the *hfq* gene used primers HfqF1 (5′-CGCCAGATGTGGTCTTACCT-3′) and HfqR1 (5′-CCTGCTCAC-CAGCATCATAA-3′). The reaction profile consisted of 30 cycles of the following in a thermal cycler (Eppendorf Mastercycler): denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s and extension at 72 °C for 2 min. Product size was confirmed by resolution on a 1% (w/v) agarose gel.

PCR products were purified directly using the Wizard PCR preps purification system (Promega Corp., Sydney, NSW, Australia) and sequence analysis of both DNA strands was performed using primers HfqF1 and HfqR1 at the Sydney University Prince Alfred Molecular Analysis Centre (Sydney, Australia). Mutations in *hfq* were identified by alignment with the known *hfq* sequence from the National Center for Biotechnology Information database, using BioEdit software (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

Fitness experiments in chemostats and batch cultures

Fitness comparisons were made against a tetracycline-resistant derivative of BW2952 carrying a *metC*::*Tn*10 insertion in medium supplemented with 4 µg ml^{-1} methionine. This strain was of equal fitness to the ancestor in glucose-limited chemostats, at all dilution rates studied. At least three chemostat competition experiments were conducted as previously described in Maharjan *et al.* (2010) and Wang *et al.* (2010), and selection coefficients were based on the equation 11 in Dykhuizen and Hartl (1983) with time measure in hours.

For fitness assay in batch cultures, exponentially growing cultures of reference and test strains in MMA supplemented with 0.2% glucose and 10 µg ml^{-1} methionine at 37 °C were mixed equally and diluted 100-fold in 30 ml of MMA plus 0.2% glucose and 10 µg ml^{-1} methionine (pre-conditioned at 37 °C). The cultures were incubated at 37 ºC with shaking 200 r.p.m. Samples were taken every hour during the exponential growth phase (at least five time points) and appropriate dilutions were plated on selective L-agar plus tetracycline (15 µg ml^{-1}) and non-selective L-agar plates. Selection coefficients of each competing strain were then calculated as in chemostat competitions.

Glucose uptake assay

For glucose transport assays, 1 ml of culture was extracted from 40-h-old chemostats and washed in 1 × MMA. Fifty microlitres of this cell suspension was mixed with 42.5 µl dH_2O, 5 µl 10 × MMA and 2.5 µl 1 µM ^{14}C-labeled glucose (Amersham, Sydney, NSW, Australia). Samples (20 µl) were taken at time points and filtered through pore size 0.45 µm filters and washed immediately with 10 ml 1 × MMA. Uptake rates were determined by measuring scintillation of ^{14}C in the cells in the culture. The optical density at 600 nm of the remainder of the washed cell suspension was measured in order to estimate the glucose uptake rate in pmol min 10^{-8} cells as described in Notley-McRobb and Ferenci (2000)).

Viable cell counts

Strains were grown in chemostat cultures at the appropriate dilution rate for 40 h before sampling. The optical density at 600 nm was measured and the samples were diluted with MMA to a final optical density$_{600}$ of 0.1. The adjusted cell suspension was diluted by a factor of 10^{-5} and plated on L-agar plates. Plates were incubated overnight and the resulting colonies were counted to obtain an estimate of the number of colony-forming cells in the chemostat culture.

Estimation of dead cells by propidium iodide staining

Samples of ~1.5 ml were removed from chemostat cultures after 40 h and cells were harvested by centrifugation. Cells were then washed once in MMA before being resuspended in 50 µl of 1 µg ml^{-1} propidium iodide (PI) solution in SSC (sodium citrate solution, 150 mM NaCl and 15 mM sodium citrate) (Sigma-Aldrich, Sydney, NSW, Australia) and incubated at room temperature for 5 min. Following staining, cells were washed once more in MMA before being spread onto a glass slide. Cells were examined using the BX51 Microscope with Reflection Fluorescence System, Mercury burner (U-RFL-T) and F-view monochrome fluorescence camera and photographed under bright-field and TxRed filters (Olympus, Tokyo, Japan). The numbers of PI-stained cells relative to total cells is a measure of dead cells (Jörgensen and Kurland, 1987) and were counted using ImageJ software (freely accessible from http://rsb.info.nih.gov/ij/)..

RESULTS

The biased selection of *hfq* mutations as a function of dilution rate

Analysis of long-term chemostat populations previously discovered evolved clones possessing mutations in *hfq* in both glucose-limited and Pi-limited chemostats (Maharjan *et al.*, 2010; Wang *et al.*, 2010); these evolution experiments used $D = 0.1\ h^{-1}$ for selection. An easily screenable phenotype of *hfq* mutations is increased sensitivity to α-MG (Maharjan *et al.*, 2010) and this phenotype was used to test the sensitivity of 320 isolates that had evolved in four slow ($D = 0.1\ h^{-1}$) and four fast ($D = 0.6\ h^{-1}$) dilution rate glucose-limited chemostat populations. After eliminating α-MG-sensitive isolates not due to *hfq* mutations by sequencing the *hfq* gene in each candidate isolate, four *hfq* alleles were found among the eight populations. There was a striking bias in the selection of *hfq* mutants, which were present in independent populations at $D = 0.1\ h^{-1}$ but not at $D = 0.6\ h^{-1}$. One isolated *hfq* allele was the same Y25D as previously characterized (Maharjan *et al.*, 2010). Other mutations resulted in S60L and L46W substitutions, along with an IS*1* insertion 18 bp upstream of the *hfq* gene (Figure 1a). All isolated *hfq* mutations reduced function of Hfq in regulating target genes such as *rpoS* (Figure 1b, altered glycogen staining with iodine, which is RpoS-dependent (Notley-McRobb *et al.*, 2002)). RpoS is the σ factor responsible for general stress

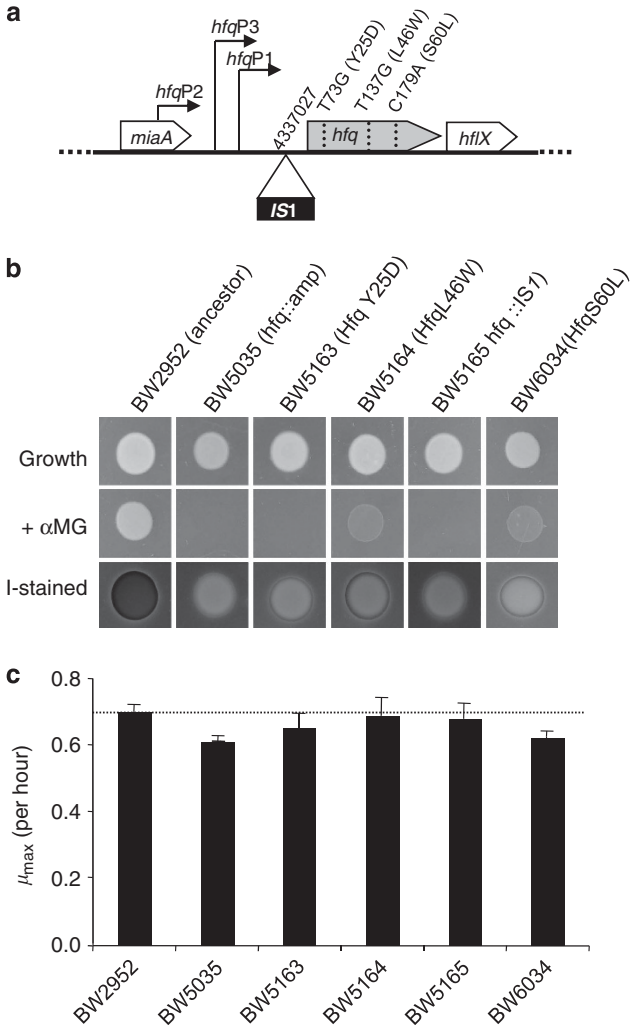

Figure 1 Phenotypic and genotype changes in four *E. coli* clones with different hfq mutations. (**a**) Schematic diagram of the BW2952 genome segment showing the location of the *IS*1 insertion in BW5165 and three separate non-synonymous mutations in three different clones. The position of IS*1* insertion in BW5165 was based on the genome position in BW2952 while the positions of point mutations indicated by the vertical dotted lines were based on the *hfq* gene sequence. Arrows with solid lines indicate positions of the three *hfq* promoters. (**b**) The phenotype was characterized in three different ways (from top to bottom): growth on glycerol MMA as a control for the sensitivity assay in the presence of methyl-α-glucoside (α-MG); sensitivity to 0.5% α-MG in a glycerol plate; staining with iodine for RpoS status is shown in the bottom row. (**c**) Maximum growth rates were measured for four *hfq* mutants and two controls, the ancestral strain and its *hfq* knockout derivative. The rate was calculated from the exponential growth phase of cultures growing aerobically in MMA containing 0.2% glucose at 37 °C. At least ten independent time points were used to estimate the growth rate. The Y-axis error bars were calculated from at least six independent experiments.

resistance, so Hfq indirectly affects many phenotypes associated with stress resistance. As expected, the mutations also affected *ptsG* function (Figure 1b) as shown by increased sensitivity to α-MG (Maharjan *et al.*, 2010). Both *rpoS* and *ptsG* are regulated by Hfq in a small-RNA-dependent manner (Gottesman, 2004). The various *hfq* mutations had broadly the same phenotypic effects, although the magnitude of these effects was dependent on the nature of the mutation (Figure 1b).

The fitness of *hfq* mutations as a function of dilution rate

In view of the pattern of selection found for *hfq* mutants, we next investigated whether the selective advantage of the *hfq* mutations was growth rate-dependent. The *hfq* alleles were individually transferred by P1 transduction into the ancestor. The resulting strains, as well as an *hfq*::*amp* knockout strain, were competed against the ancestral strain in glucose-limited chemostats over a sixfold range of dilution rates. In this study, growth rate is measured as μ, equivalent to r in Malthusian growth models, and called the specific growth rate in bacterial growth equations. When growing in chemostats, μ is set by the dilution rate D, and $D=\mu$ at pseudo-steady state (Ferenci, 2007), so different growth rates were experimentally established by setting D between 0.1–0.6 h^{-1}, equivalent to doubling times between 69 min and nearly 7 h.

The different evolved *hfq* mutations all provided positive fitness at $D=0.1\,h^{-1}$ (Figure 2), explaining the selection of the mutants in evolving 0.1 h^{-1} populations. The constructed *hfq* knockout strain, however, was not fitter at any dilution rate. This suggests that residual Hfq activity is needed for some cellular functions; complete loss of function mutations were indeed not selected in chemostats.

Interestingly, the selection coefficient of each of the *hfq* mutants was found to vary markedly with changing dilution rate (Figure 2). Most strikingly, the measured selection coefficients all changed sign at some point as dilution rate increased, although the position and speed at which this occurred differed between alleles, indicative of antagonistic pleiotropy. Particularly the point mutations Y25D and S60L showed a drastic change in fitness, sharply decreasing as dilution rate approached $D=0.6\,h^{-1}$. In competitions at the maximal growth rate (μ_{max}) in batch culture (batch culture because chemostats do not allow competition at μ_{max}, as cells are washed out), relatively small differences between mutants and wild-type were found.

Another interesting result was that the range of fitness effects with the set of evolved alleles differed with dilution rate. As indicated by the s.d's in Figure 3a at each growth rate, the five different alleles showed the most divergent fitness effects at low dilution rates but converged and were more similar at fast dilution rate and at the μ_{max}. As further shown in Figure 3b, particular alleles were more prone than others to have a wide range of s values at different growth rates. So the net fitness effects of *hfq* mutations are growth-rate affected, although the small sample of mutations in a single gene limits conclusions for fitness distributions in general.

The molecular basis of fitness as a function of dilution rate

We attempted to define both the beneficial and deleterious effects of *hfq* mutations that varied with growth rate. Two *hfq* alleles that showed different fitness profiles (*hfq*Y25D and the *hfq* knockout strain) were chosen to observe variation in phenotypic effects across three dilution rates ($D=0.1$, 0.4 and 0.6 h^{-1}).

First tested was glucose uptake rate, which is the main fitness determinant in glucose-limited chemostats (Ferenci, 2007); this was measured by following the uptake of ^{14}C-glucose by the *hfq* mutants and the ancestor (Figure 4a). At the beneficial dilution rate of $D=0.1\,h^{-1}$, both *hfq* mutants show a significantly higher glucose uptake rate than the ancestor (two-tailed t-test $P=0.008$). A major contributor to this difference is the elevated transport through PtsG in *hfq* mutants (Maharjan *et al.*, 2010). The transport difference is smaller but still positive at $D=0.4\,h^{-1}$. At $D=0.6\,h^{-1}$, the glucose uptake rate of the ancestor and the *hfq*Y25D mutant is not significantly different (two-tailed t-test $P=0.77$), however, the *hfq* knockout mutant shows a lower glucose uptake rate than either (two-tailed t-test $P<0.005$ for both strains). The convergence of uptake

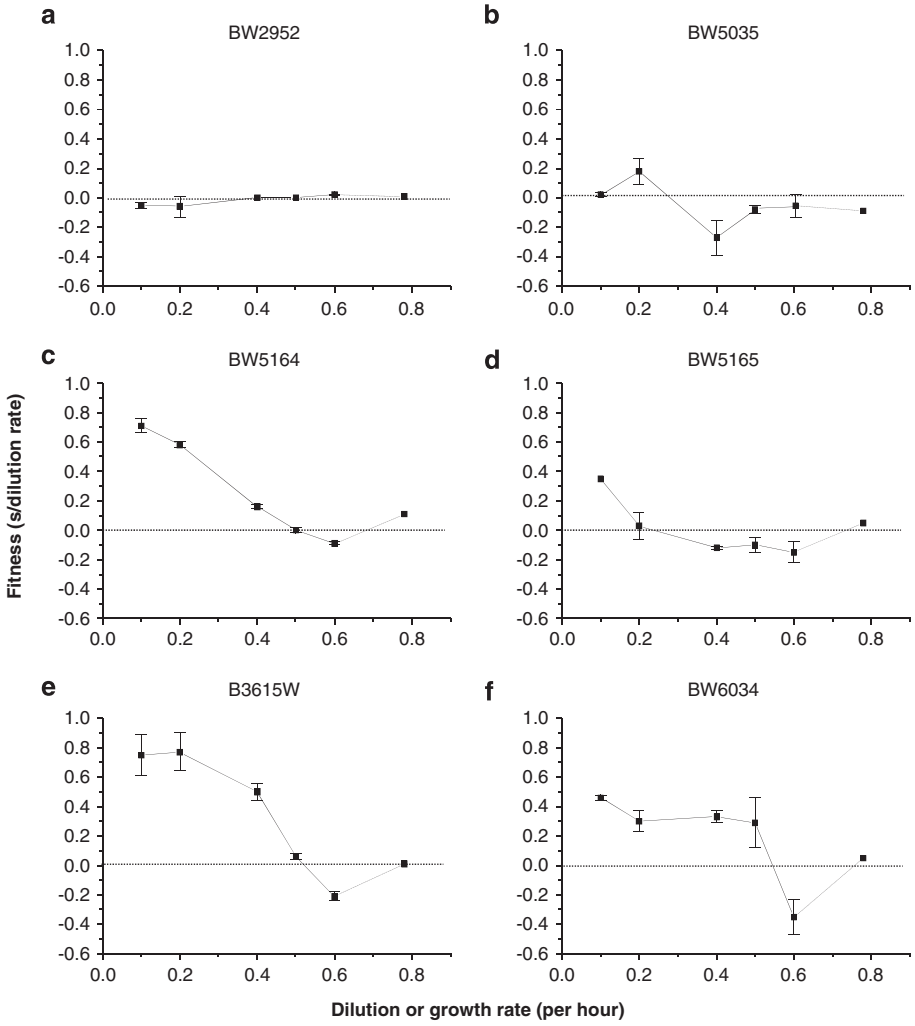

Figure 2 Effects of hfq mutations on competitive fitness and maximum growth rate. Competitive fitness was measured for strains bearing the shown mutant *hfq* alleles in glucose-limited chemostats (Ferenci, 2007). Competitions were against a reference strain BW3454, which is the ancestral strain but containing a *met*C::Tn10 countable marker. This reference strain was neutral in fitness when competed against the ancestor as shown in (**a**). Competing strains were mixed to an ~50:50 ratio after 16 h individual acclimatization at the appropriate dilution rate. In addition to chemostat experiments, the same strains were also competed in glucose-excess batch culture at the $\mu_{max,}$ which cannot be achieved in chemostats; the results in batch culture are plotted joined by dotted lines. Changes in population proportions were used to calculate selection coefficients (Dykhuizen and Hartl, 1983). To eliminate the effect of different generation times, the data is presented as s/μ (Chevin, 2011). The mean and s.d.'s were obtained from three replicate experiments. The different *hfq* mutants are shown independently in (**b**) *hfq*::amp knockout, (**c**) L46W mutant (**d**) IS*1* insertion in *hfq* promoter, (**e**) Y25D mutant and (**f**) S60L mutant. Dashed lines between $D=0.6$ and $0.8\,h^{-1}$ indicate the competitions performed at μ_{max} or batch cultures.

rates and the major benefit being exhibited at slow growth rate can be explained to be due to two previously determined factors in patterns of gene regulation. In addition to the *ptsG* effect, Hfq contributes to the high RpoS levels in ancestral bacteria at $D=0.1\,h^{-1}$, and reduced Hfq function removes the negative effect of RpoS on transport (Maharjan *et al.*, 2010). RpoS is a lesser negative influence at the higher growth rate because it is not so highly expressed (Notley and Ferenci, 1996). In addition, wild type cells naturally optimize their transport at $D=0.6\,h^{-1}$ by inducing an alternative Mgl transport system (Ferenci, 1996).

Consistent with the above explanation, the differential effect of dilution rate on the expression of glucose transporters is illustrated by the level of a *malG*::*lacZ* reporter gene fusion. The *mal* genes are known to regulate expression of the outer membrane glycoporin *lamB* (Notley and Ferenci, 1995), which is involved in glucose uptake at limiting glucose concentrations (Death *et al.*, 1993). The *malG*::*lacZ* fusion is also indirectly regulated by *hfq* (Maharjan *et al.*, 2010) β-galactosidase assays were carried out on chemostat cultures of the ancestor and both *hfq* mutants at the three dilution rates tested above (Figure 4b). At $D=0.1\,h^{-1}$, β-galactosidase activity was significantly higher in both *hfq* mutants (two-tailed *t*-test $P<0.002$ for both strains) than in the ancestor strain, which shows very low levels of expression under these conditions. At this dilution rate, β-galactosidase activity in the Δ*hfq* mutant is also significantly higher than the *hfq*Y25D mutant (two-tailed *t*-test $P=0.001$). At $D=0.4\,h^{-1}$, *malG* expression is significantly higher for both the ancestor and the *hfq*Y25D mutant than at 0.1 (two-tailed *t*-test $P<0.002$, $P=0.004$, respectively), while levels of expression in the *hfq* knockout mutant converge with those for *hfq*Y25D (two-tailed *t*-test $P=>0.05$). At $D=0.6\,h^{-1}$ however, there is no significant difference between either of the *hfq* mutants and the ancestor (two-tailed *t*-test $P>0.05$ for both strains). The high expression of transporters like LamB protein

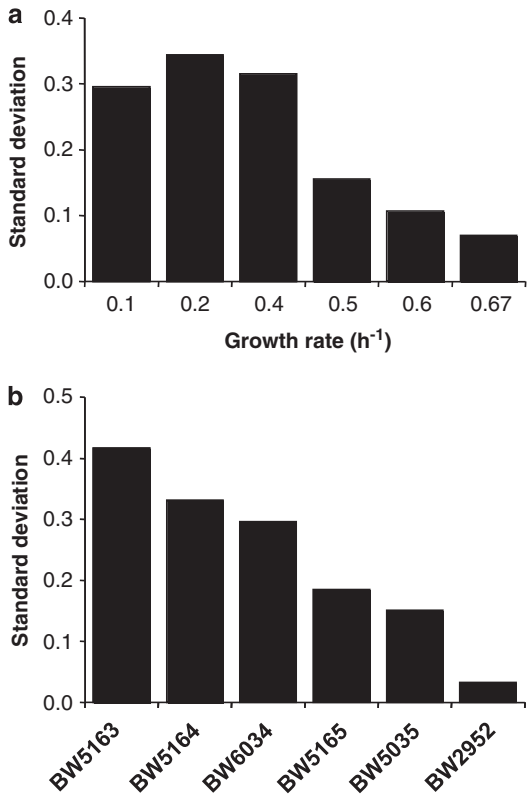

Figure 3 The variance of fitness effects in hfq mutants at different growth rates. In (**a**), the fitness estimates from Figure 2 were used to calculate s.d.'s at each given dilution rate, using the values from the four independently evolved isolates. In (**b**) the s.d. was measured for each allele, with the fitness values included from all growth rates.

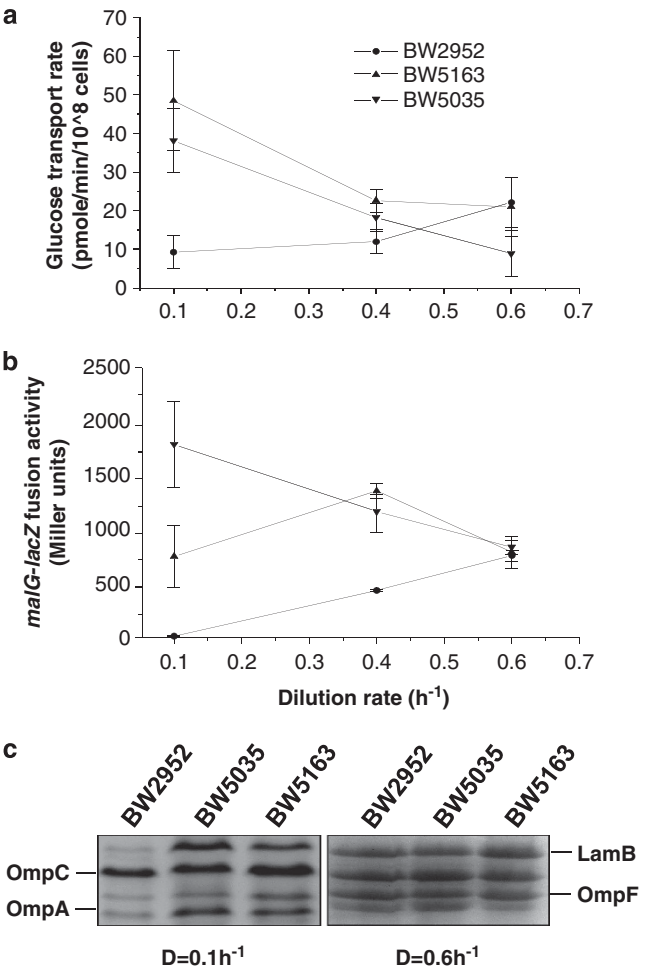

Figure 4 Changes of nutrient uptake with dilution rate in hfq mutants. Ancestor strain BW2952, Δ*hfq* mutant BW5035 and *hfq*Y25D mutant BW5163 were cultured in chemostats for 40 h at dilution rates of $0.1\,h^{-1}$, $0.4\,h^{-1}$ and $0.6\,h^{-1}$. Assays were then performed to determine: (**a**) Glucose transport rate as measured by the uptake of radiolabelled ^{14}C-glucose (Notley-McRobb and Ferenci, 2000); (**b**) Expression of *malG*::*lacZ* gene fusion as measured by β-galactosidase activity (Notley and Ferenci, 1995). (**c**) Outer membrane protein profiles of the ancestor strain BW2952, Δ*hfq* mutant BW5035 and *hfq*Y25D mutant BW5163 at $0.1\,h^{-1}$ and $0.6\,h^{-1}$ were analyzed as described in Maharjan (2006). Means and s.d.'s were obtained from 3–7 biological replicates.

relative to other major outer membrane proteins at $D=0.6\ h^{-1}$ is also shown in Figure 4c, comparing equal numbers of extracted cells. At this dilution rate, the *hfq* mutations have little influence on the LamB level. In contrast, at $D=0.1\,h^{-1}$, the ancestor has repressed LamB and the *hfq* mutations elevate LamB levels to that seen at $D=0.6\,h^{-1}$. Overall, the difference in transport benefit due to the *hfq* mutations is highly dilution rate-dependent because the cellular context is markedly different between $D=0.1$ and $0.6\,h^{-1}$, as shown by all the results in Figure 4.

The deleterious effects of the *hfq* mutations were also investigated. Previous studies have shown that a mutation in *hfq* causes cell division errors (Tsui *et al.*, 1994) and reduced cell viability (Večerek *et al.*, 2008). To understand if the deleterious fitness effect of *hfq* mutations at $D=0.6\,h^{-1}$ was due to increased cell death in the population, we measured the proportion of live and dead cells after staining with PI across the three dilutions rates ($D=0.1$, 0.4 and $0.6\,h^{-1}$). As shown in Figure 5, dead cells in populations of both Y25D and Δ*hfq* mutants were more common, compared with ancestor at $D=0.6\,h^{-1}$. Also at $D=0.6\,h^{-1}$, we found the proportion of PI staining was around 11 and 19% compared with that of 5 and 6% at $D=0.1\,h^{-1}$ for Y25D and the *hfq* knockout strain, respectively, (two-tailed *t*-test $P=0.019$, 0.023, respectively). On the other hand, the PI assays indicated that the viability of the ancestral strain remained unchanged with all the three dilution rates (Figure 5) so the dilution rate-dependent decrease in viability could contribute to the reduced fitness of the *hfq* mutants at $D=0.6\,h^{-1}$. However, the viability drop between $D=0.4$ and $0.6\,h^{-1}$ was less drastic than the fitness shift in Figure 2, so there may well be other deleterious effects of the mutations. Still, the transport advantage at $D=0.6-h^{-1}$ and the viability disadvantage at $D=0.6\,h^{-1}$ are likely contributing factors to the antagonistic pleiotropy as a function of growth rate.

DISCUSSION

Several alleles of *hfq* were enriched in independent glucose-limited populations in <100 generations, so the parallelism is indicative of a reproducible selective advantage under nutrient limitation. Each of the alleles affected functions independently controlled by different small RNAs, such as *rpoS* and *ptsG* expression, as well as porin levels (Figures 1 and 4). We observed no null mutations in *hfq*, but the promoter IS mutation suggests that reduced Hfq protein amount can also be a benefit at slow growth rates. An artificially constructed *hfq* knockout had no selective advantage, explaining the absence of null

mutants from evolving populations. This also suggests that some Hfq protein or partially functioning protein is a benefit to a cell. Given the multiplicity of *hfq* functions, from histone-like activity to cell division and RNA degradation (Tsui *et al.*, 1994; Gottesman, 2004), this is not altogether surprising.

The selective advantage of the various point mutations varied under the common selection condition (dilution rate $0.1\,h^{-1}$) so there was a range of fitness effects in mutations within the same gene at the same growth rate. The Y25D substitution had the greatest mutational effect, phenotypically and in terms of benefit, followed by L46W and S60L. These amino acid residues are located in Hfq towards one face of the donut-shaped hexameric protein, as shown in Figure 6. The substitutions occur away from the poly-U binding face and closer to the distal face shown to preferentially bind poly-A RNA and *rpoS* mRNA (Mikulecky *et al.*, 2004; Link *et al.*, 2009). We have not undertaken RNA-binding studies, but the Y25D substitution was fortuitously studied in a site-directed mutagenesis analysis, and shown to reduce *rpoS* mRNA binding (Mikulecky *et al.*, 2004). An S60A substitution was also studied (Mikulecky *et al.*, 2004), but our S60L mutant has a greater effect on *rpoS* expression, probably because of the less conservative substitution involved.

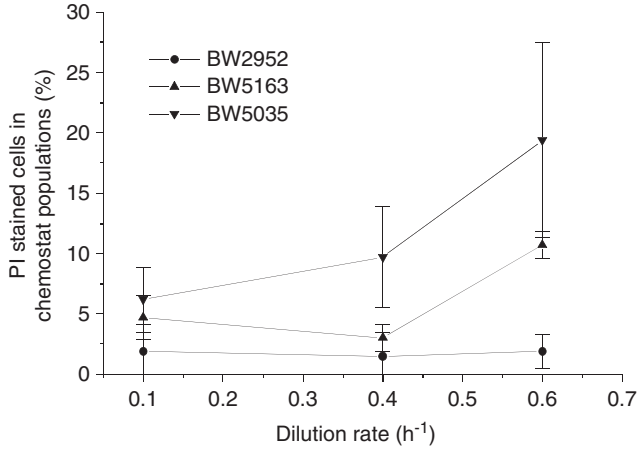

Figure 5 Changes of cell viability with dilution rate in hfq mutants. Ancestor strain BW2952, Δ*hfq* mutant BW5035 and *hfq*Y25D mutant BW5163 were cultured in chemostats for 40 h at dilution rates of $0.1\,h^{-1}$, $0.4\ h^{-1}$ and $0.6\,h^{-1}$. Assays were then performed to determine live- and dead-cell staining with PI. Means and s.d.'s were obtained from 3–7 biological replicates.

The precise molecular mechanism of the negative contribution to the observed antagonistic pleiotropy present in *hfq* mutants has not been identified so far. However, the differential effects of *hfq* mutations on death rate at different growth rates is a likely contributor to the negative fitness at $D = 0.6\,h^{-1}$. One identified beneficial effect of the *hfq* mutations is on transport, at least between dilution rates of $0.1\,h^{-1}$–$0.4\,h^{-1}$. This and other potential beneficial effects of *hfq* mutations (Maharjan *et al.*, 2010) are most evident at $D = 0.1\,h^{-1}$, the original selection condition. At high dilution rates ($D = 0.6\,h^{-1}$) the transport advantage is reduced and especially between $0.4\,h^{-1}$and $0.6\,h^{-1}$, a deleterious effect due to decreased viability cuts into decrease fitness. Further work is needed to identify the precise molecular mechanism of increased death rates, which appear between $D = 0.1\,h^{-1}$ and $D = 0.6\,h^{-1}$.

There are several important implications of this demonstration with *hfq* mutants that *s* can be extremely sensitive to slight differences in growth conditions. Firstly, as a demonstration of antagonistic pleiotropy, it is an interesting result that a mutation in *hfq* has both beneficial and deleterious effects simultaneously, each of which varies with the growth rate. An explanation of antagonistic pleiotropy from an ecological perspective is that the growth rate is determined in chemostats by the residual glucose concentration, in other words, resource availability. So the difference in the *hfq* mutants is between good competitive ability at $D = 0.1\,h^{-1}$, with low resource availability, and $D = 0.6\,h^{-1}$, where the residual glucose is higher in concentration (Ferenci, 2007).

A second implication of these results is for the estimation of the magnitude of *s* in a variety of mutation analysis experiments, which generally involve competition with ancestor under a variety of conditions (Elena and Lenski, 2003; Perfeito *et al.*, 2007; MacLean and Buckling, 2009; Lind *et al.*, 2010McDonald *et al.*, 2011). Fitness assays, especially in experimental evolution studies, use the selection conditions for competitions between ancestor and evolved strains. In the Lenski experiments for example, relative fitness is measured over a 24-h period in a batch culture. The bacteria experience various growth phases and growth rates in such an assay. The temporal fluctuations within a 24-h period allow fitness to evolve in parts of the 24-h period (Rozen *et al.*, 2009). It is not clear how many of the

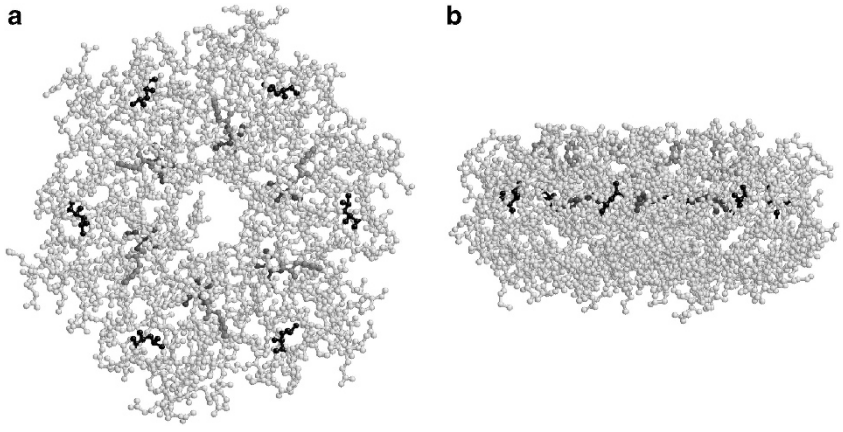

Figure 6 Structural changes in Hfq evolved in chemostat populations. Structure of the hexameric form of Hfq protein showing the sites of the residues altered in the evolved mutants. Residue Y25 is in red, L46 in black and S60 in blue, all positioned away from the distal face binding poly-A RNA and closer to the proximal face binding small-RNAs (Link *et al.*, 2009). (**a**) view from the small-RNA-binding face and (**b**) side on. A full color version of this figure is available at the *Heredity* journal online.

global regulatory mutations found in the Lenski populations (Philippe *et al.*, 2007), or indeed in any experimental population studied by other groups, is subject to growth rate effects on fitness.

The distribution of fitness effects of mutations has been the subject of many studies (Barrett *et al.*, 2006; Hegreness *et al.*, 2006; Kassen and Bataillon, 2006; Perfeito *et al.*, 2007; MacLean and Buckling, 2009; Lind *et al.*, 2010; McDonald *et al.*, 2011). Interestingly, the range of fitness effects for the *hfq* alleles studied here is quite different under slightly different conditions for testing fitness at the various dilution rates. As shown by the s.d.'s at different dilution rates (Figure 3a), there are growth rates at which the different alleles have similar fitnesses but others where there is significant variation between alleles. Also, particular alleles are more prone to have variable *s* values at different growth rates. Here again, we need to invoke differential changes in the mutants altered at different positions in Hfq and the interaction with multiple protein and RNA partners to explain this phenomenon. Although this variance may be restricted to pleiotropic regulatory mutations such as in *hfq*, it does raise a more general question as to the plasticity of fitness distributions in general.

An important reason for estimating *s* is to predict the fixation of mutations in an evolving population. The results presented here suggest that the growth rate difference between ancestor and an evolved strain with a beneficial mutation can strongly affect the benefit over the course of fixation. In a selection environment like the chemostat, it is likely that the fixation trajectory of each *hfq* mutation in the *E. coli* populations is complicated by the changing growth rate advantage of the mutants. If initially a population is growing at a $0.1\,h^{-1}$ dilution rate in the selection environment, a newly mutated *hfq* strain with better nutrient uptake has a growth rate advantage over the majority ancestral population when growing on the available residual glucose. We do not know the residual glucose concentration when the strains evolved or the growth rate when rare, but if the available glucose permits, for example, a growth rate μ of $0.2\,h^{-1}$, then the mutant will have a strong positive benefit. If, however, the initial residual glucose allows a μ of $0.5\,h^{-1}$, then the data in Figure 2 suggests that the advantage of the *hfq* mutant is reduced, so is effectively subject to negative feedback with higher growth rate. If the mutants increase in proportion and the population eventually consists of a majority of evolved bacteria, these reduce residual glucose levels so μ is gradually reduced towards 0.1 (the set dilution rate). Speculatively, the non-constant benefit over the trajectory of fixation can look like frequency dependence; when in low abundance, the mutant will be subject to negative feedback because of the potential for increased growth rate, which will be relieved later at higher mutant abundance when the growth rate approaches the set dilution rate. Further study is required to establish whether the growth rate dependence of fitness can be an explanation of frequency dependence without outside ecological interactions. If so, this would be an important explanation of a wide range of phenomena where frequency dependence is either observed or postulated.

In summary, we demonstrated a series of distinct growth rate vs *s* profiles for the alleles of the same gene, because of antagonistic effects involving the pleiotropic *hfq* gene. Firstly, in a glucose-limited environment we find the benefit comes from improved glucose transport, but this is restricted to slow growth conditions. At faster growth rates, this benefit disappears, but an increasing defect in cell viability caused by the *hfq* mutations results in negative fitness. The variation in *s* demonstrated in the same environment but at different growth rates with *hfq* mutations has relevance to *s* estimations, description of the fitness distribution of mutations, as well as frequency-dependent behavior in the absence of obvious ecological interactions.

DATA ARCHIVING

Data deposited in the Dryad repository: doi:10.5061/dryad.5bd50.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Luis-Miguel Chevin for helpful comments on an earlier version of the manuscript and the Australian Research council for funding support.

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