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# Variation in the propensity to diversify in experimental populations of *Escherichia coli*: consequences for adaptive radiation

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## ABSTRACT

**Question:** Does genotype regulate diversification during adaptive radiation?

**Hypothesis:** Experimental populations initiated from different genotypes vary in their propensity to undergo analogous adaptive radiations in the same environment.

**Organisms:** We used slow-switcher and fast-switcher genotypes (reflecting different specialized metabolic phenotypes) derived from the bacterium *Escherichia coli* B.

**Methods:** In a previous evolution experiment, we observed the generation of novel, specialist genotypes (slow- and fast-switchers) evolved under adaptive radiation. We isolated these genotypes and assessed their propensity to further evolve and diversify in the same evolutionary conditions that gave rise to them. Specifically, we wished to determine whether these genotypes could recapitulate the original diversity produced during the first bout of adaptive radiation. We assessed metabolic diversification using kinetic growth profiles.

**Conclusions:** Slow-switchers could recapitulate the original diversity, while fast-switchers never did. Thus, some genotypes appear constrained in their ability to generate mutations that would otherwise be selected during adaptive radiation. This demonstrates the importance of variational input as a regulator of diversification.

*Keywords*: adaptive radiation, character displacement, diversification, *Escherichia coli*, growth curve analysis, metabolism, mutational bias.

*[U]nless profitable variations do occur, natural selection can do nothing*.
(Darwin, 1859)

## INTRODUCTION

Adaptive radiation is an evolutionary process that transforms one species into an array of species each having a distinct phenotype (Simpson, 1953; Schluter, 2000). Extrinsic factors, such as divergent selection and ecological opportunity, have been identified via natural and experimental studies to be important drivers of adaptive radiation (reviewed in Schluter, 2000). This

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emphasis on extrinsic factors – those factors that are external to the individual organism – has culminated in a successful 'ecological theory' (Schluter, 2000) to explain the origin of variation during adaptive radiation. In contrast, intrinsic factors, such as mutation and development [or 'mutation-as-altered-development' (Stoltzfus, 2006)], have received less attention from evolutionary ecologists studying adaptive evolution (but see West-Eberhard, 2003; Brakefield, 2006). There are at least two reasons for considering intrinsic factors in adaptive radiation. First, a consideration of intrinsic factors may explain what forms arise under adaptive radiation, and serve to complement explanations for why those forms persist. Second, mutation is the only evolutionary force that introduces novel adaptive phenotypes to a population (i.e. increases the frequency of an allele from zero to above zero). Thus, differences in mutation rates among species may result in the asymmetric introduction of novel phenotypes, which may cause divergent outcomes among evolving lineages even in the face of convergent opportunities and convergent patterns of selection. Ultimately, asymmetries in intrinsic factors may explain why some species leave more descendent species than others when ecological opportunity and selection are similar (Kassen, 2009).

In this paper, we characterize variation in the propensity to diversify and its consequences for adaptive radiation. Using a model adaptive radiation of *Escherichia coli* (Le Gac *et al.*, 2008; Spencer *et al.*, 2008; Tyerman *et al.*, 2008), we examined whether there was variation between ecotypes (i.e. species) in propensity to diversify with respect to metabolic traits. Specifically, we isolated two phenotypically divergent ecotypes from each of three diversified populations. Next, we initiated new populations from each ecotype and propagated them for 200 generations under the same ecological (i.e. extrinsic) conditions that caused adaptive radiation in their recent ancestor. We have previously analysed changes in mean metabolic traits within these newly derived populations (Tyerman *et al.*, 2008). Here, we characterize variation in metabolic phenotypes within these populations and then contrast the level of variation among populations.

By isolating divergent ecotypes, we reduced interspecific competition for resources (Tyerman *et al.*, 2008) and thereby increased the ecological opportunity (Schluter, 2000). Our expectation was that this would promote continued adaptive radiation. In addition, because the isolated ecotypes experienced analogous ecological conditions as their ancestor, we had an *a priori* expectation for the extent and form of variation possible in our experimental system that we used as a baseline hypothesis. Thus, in controlling extrinsic factors (e.g. ecological opportunity), we were able to experimentally test whether intrinsic factors could affect the extent and form of diversity evolved during subsequent adaptive radiation.

Variation in mutational input, resulting from intrinsic variation (e.g. in genetic background), may exacerbate the role of historical contingency [i.e. the series of (mutational) events experienced by different species]. Contingency is often described as a stochastic element in evolutionary scenarios, in contrast to deterministic processes (i.e. selection) (Travisano *et al.*, 1995; Losos *et al.*, 1998; Gould, 2002). Yet, mutational contingency may nonetheless be biased, with the result that some lineages may be predisposed to access regions of phenotype space not available (or, with reduced availability) relative to other lineages. Whether variation in accessibility contributes to variation in patterns of diversity under adaptive radiation is not traditionally considered by theory. Rather, theory often assumes that the production of phenotypic variation is isotropic (i.e. equivalent in all directions). However, when theory has explicitly allowed variation in genetic opportunity, it has been shown to direct adaptive evolution (Yampolsky and Stoltzfus, 2001).

The proliferation of phenotypes during adaptive radiation is an important topic in evolutionary biology, yet most research has focused on ecological opportunities and patterns of selection that sort phenotypic variants in diverging populations. In this study, we test for mutational bias (an intrinsic factor) and its impact on the diversity produced under adaptive radiation. Our data show that ecotypes vary in the propensity to diversify, leading to significant differences in diversity produced under adaptive radiation. These results suggest that mutational constraints may be an important factor in shaping evolutionary outcomes.

## METHODS

### Background

The bacterial strains used in this study were isolated from the 1000-generation time point of a long-term evolution experiment (Le Gac *et al.*, 2008; Spencer *et al.*, 2008; Tyerman *et al.*, 2008). In that long-term evolution experiment, we propagated *Escherichia coli* B following a seasonal (or 'batch') protocol (see Lenski *et al.*, 1991; Spencer *et al.*, 2007b). We supplemented the medium with glucose and acetate as the sole carbon sources (after Friesen *et al.*, 2004). In ten of ten replicate populations, we observed parallel patterns of within-culture (i.e. sympatric) diversification with respect to metabolic function (Tyerman *et al.*, 2008).

To understand this metabolic diversity, we briefly review resource consumption in *E. coli*. When *E. coli* is propagated in batch culture with mixed resources, these resources are consumed sequentially (reviewed in Harder and Dijkhuizen, 1982). Bacteria supplemented with glucose and acetate typically (i.e. ancestral condition) consume glucose exclusively until exhausted from the medium, and then switch to consuming acetate. Metabolism is thus developmentally flexible (i.e. phenotypically plastic), as the expression of acetate metabolizing genes is repressed in environments with sufficient concentrations of glucose. The ancestor is therefore sensitive to the (glucose) environment. This environmental sensitivity that takes the form of a metabolic inhibition is called 'catabolite repression'. Catabolite repression likely evolved because of functional trade-offs between metabolizing glucose and other carbon sources (e.g. acetate). This pattern of metabolic regulation results in a two- phase or diauxic growth profile (Fig. 1). Glucose is consumed during the first phase and acetate is consumed during the second phase. Previous studies have demonstrated that genetic changes can modify metabolism such that growth profiles are shifted (Spencer *et al.*, 2007a). Thus, we measure shifts in growth profile as a quantitative measure of metabolic evolution (Tyerman *et al.*, 2008).

After 1000 generations of evolution, we commonly observed two co-existing ecotypes in our evolved populations. An ecotype is a phenotype that correlates with some aspect of ecology experienced by that phenotype (in our case, resource availability and metabolic strategy). One ecotype – the slow-switcher – was characterized as having a long diauxic lag in transitioning from glucose to acetate growth. A second ecotype – the fast-switcher – had a negligible diauxic lag in transitioning from glucose to acetate consumption (Fig. 1). Slow-switchers have evolved stronger catabolite repression than the ancestor (Spencer *et al.*, 2008). Thus, slow-switchers are highly sensitive to the glucose environment. Fast-switchers, on the other hand, appear to be free from catabolite repression, and may consume acetate constitutively (Spencer *et al.*, 2007a). Using a microarray study, Le Gac *et al.* (2008) described how expression profiles changed during the course of the long-term evolution experiment,

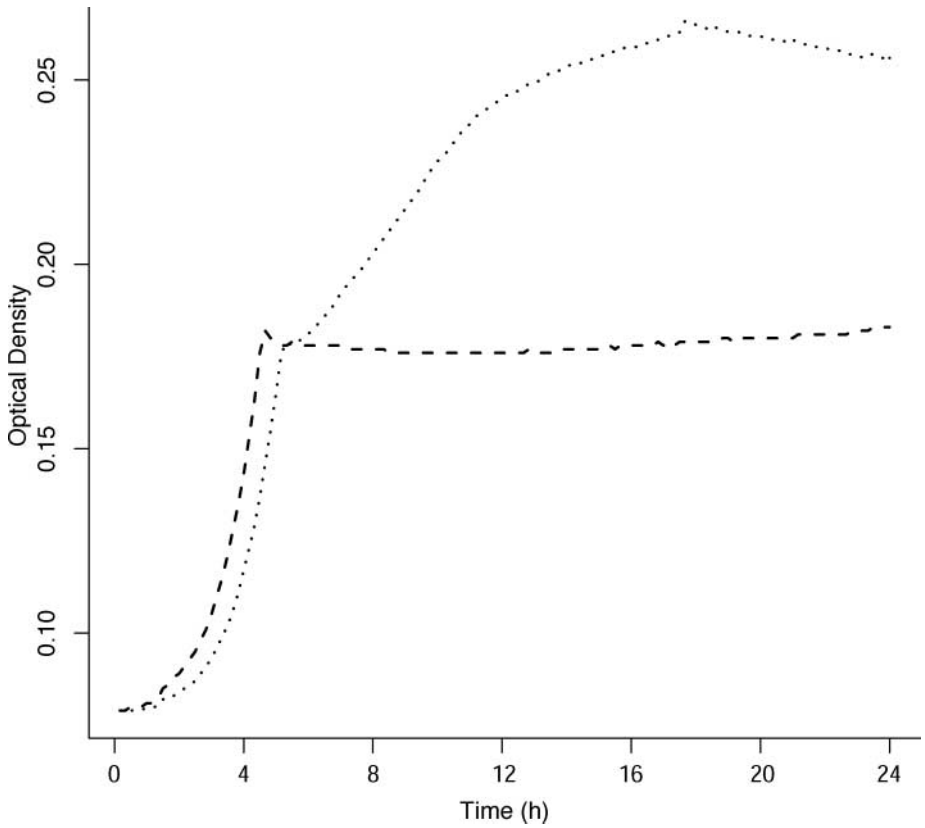


**Fig. 1.** 24-h growth curves for source ecotypes from source population 18 (strain dst1018). Slow- and fast-switcher ecotypes are shown by dashed and dotted lines, respectively.

indicating that both the slow- and fast-switcher ecotypes had changed relative to their ancestor, and relative to each other.

## Isolation treatment

We have previously reported on changes in mean trait levels that occurred in populations evolved from each ecotype in isolation (Tyerman *et al.*, 2008). Here, we focus on the diversity evolved within these populations by characterizing individual clones isolated from a subset of these populations.

First, we provide an overview of our experimental design and expand on the details below. From the 1000-generation point of our long-term evolution experiment, we selected three of ten diversified populations (strains: dst1018, dst1019, and dst1020, hereafter referred to as 18, 19, and 20 respectively) to serve as 'source populations. From each source population, we isolated and selected five slow-switcher and five fast-switcher clones (hereafter referred to as 'source ecotypes') to serve as founding populations in this study. Thus we had three diversified source populations × two source ecotypes × five clones per source ecotype per source population = 30 founding populations. We evolved the founding populations for 200 generations. After 200 generations, we referred to the 30 populations as

'derived populations' and to individual clones isolated from derived populations as 'derived ecotypes'. We elaborate these details below.

We maintained our source populations at −80°C in glycerol. Our selection of three source populations (from a possible ten diversified populations) in this study was based on preliminary analyses of diversity in these strains (Tyerman *et al.*, 2008). From frozen stock, we inoculated each source population into fresh medium and conditioned for 24 h at 37°C and 250 rev · $min^{-1}$, following the conditions used in the long-term evolution experiment (Tyerman *et al.*, 2008). We plated these cultures on agar, and arbitrarily selected five clones of each ecotype based on differences in colony morphology [slow-switchers having large colonies; fast-switchers having small colonies (Friesen *et al.*, 2004)]. We verified that the selected clones had appropriate growth curve characteristics for the anticipated source ecotype (slow- or fast-switcher) and stored these clones at −80°C in glycerol.

We initiated 30 founding populations by inoculating frozen stock of each clone into fresh medium for 24 h (as above), which gave rise to isogenic cultures (assuming no mutation). These cultures served as the sources for our founding populations. We inoculated 1.5 $\mu$l of stationary phase founding population in 150 $\mu$l of media into individual wells of a 96-well microplate. We propagated these populations daily (transferring 1/100 of population once every 24 h into fresh media) for 30 days. This yielded approximately 200 generations of evolution for each population after isolation from its diversified source population. Otherwise, conditions were analogous to the original long-term evolution experiment.

After 30 days, derived populations were plated on agar and we randomly selected ~92 clones per derived population to characterize evolved phenotypic variation.

## Growth curve profiles

Following Tyerman *et al.* (2008), we characterized the metabolic phenotypes of the derived clones. [To clarify, Tyerman *et al.* (2008) characterized the mean metabolic phenotype of derived populations; here, we characterized the metabolic phenotypes of variants isolated from derived populations.] Briefly, we conditioned culture from each derived clone and inoculated 1.5 $\mu$l in 150 $\mu$l of fresh medium into an individual well of a 96-well plate, and incubated using Biotek shaking incubators that periodically measured optical density. Growth curves were created by measuring optical density (600 nm) at 10-min intervals over 24 h of incubation. We used a computer program to extract relevant growth curve parameters for each clone. Briefly, we extracted ten growth curve parameters that captured variation in diauxic growth performance, including growth lag, maximum growth rate, and yield for each growth phase. These growth curve parameters capture variation among clones that have diversified with respect to how they transition from first to second growth phase during diauxic growth. Further details are provided in Tyerman *et al.* (2008).

## Statistical analysis

All analyses were conducted with R, version 2.6.2 (R Development Core Team, 2008). For each growth curve, ten growth parameters were extracted (see Tyerman *et al.*, 2008) and log-transformed to correct for scaling. For each derived population, we conducted a principal components analysis (PCA) on the correlation matrix of its derived clones, and generated 10 composite traits (Tyerman *et al.*, 2008). Using these composite traits, we conducted a hierarchical cluster analysis with hclust (Euclidean distance, 'ward' method) detailed in the package cluster

(Maechler *et al.*, 2005). We conducted bootstrap analyses using pvclust (Suzuki and Shimodaira, 2006) to determine *P*-values for nodes of the branching points for all dendrograms (nboot = 10,000 randomizations). We used a threshold of five distance units to discriminate among clusters within derived populations. This allowed us to compare diversity across derived populations. The criterion of five units reflected a balance between choosing clusters that had support based on *P*-values (≥80%) and having ≥5 individuals. We called these clusters 'ecotypes' and hereafter refer to them as 'derived ecotypes' to distinguish them from the source ecotypes (i.e. slow- or fast-switcher) that were used as the progenitors to the founding populations. To analyse the range of phenotype space accessed by derived populations, we conducted a hierarchical cluster analysis on the set of derived ecotypes from all derived populations. For derived ecotypes, we used the mean derived ecotype, rather than the collection of individuals. As above, we used a threshold of five distance units to discriminate between clusters. We conducted analyses of variance (ANOVA) to determine whether variation in two response variables, extent and range of radiation, could be explained by variation in source population, source ecotype, and the interaction term. Extent of radiation was a comparison of the number of ecotypes evolved in each derived population. Range of radiation was the amount of phenotype space accessed by each derived population, using a common projection (see description in Results).

## RESULTS

### Extent of radiation

We determined the number of significant clusters (e.g. 'derived ecotypes') in each population (see Methods). Next we conducted ANOVA to determine whether source population (populations 18, 19, and 20) and source ecotype (slow-switcher and fast-switcher) explained variation in numbers of derived ecotypes among derived populations (Table 1). Source population had a marginal though insignificant role in explaining the observed variation ($F_{2,24} = 2.84,\ P = 0.078$), while source ecotype (slow-switcher or fast-switcher) strongly affected the subsequent extent of radiation ($F_{1,24} = 12.29,\ P < 0.0018$). As there was no significant interaction between source population and source ecotype in explaining variation in extent of radiation ($F_{2,24} = 1.38,\ P = 0.27$), we grouped data from different source populations, and calculated the mean number of derived ecotypes in slow-switcher and fast-switcher derived populations. On average, slow-switcher populations diversified into 4.9 (95% confidence interval: 4.0 to 5.8) derived ecotypes and fast-switcher populations diversified into 3.1 (95% confidence interval: 2.4 to 3.9) derived ecotypes.

**Table 1.** Summary of ANOVA conducted on extent of adaptive radiation (i.e. number of derived ecotypes) after 200 generations of evolution under competitive release

| Source of variation | d.f. | SS | MS | *F* | Pr(>*F*) |
|---|---|---|---|---|---|
| Population | 2 | 10.4 | 5.2 | 2.84 | 0.078 |
| Ecotype | 1 | 22.5 | 22.5 | 12.29 | **0.0018** |
| Population × ecotype | 2 | 5.1 | 2.5 | 1.38 | 0.27 |
| Residuals | 24 | 44.0 | 1.8 | | |

## Range of radiation

We transformed all derived ecotypes from all derived populations using a common phenotypic mapping, first described in Tyerman *et al.* (2008). This mapping illustrated phenotypic convergence along $Z_{PC1}$ (and parallel phenotypic shifts along $Z_{PC2}$) that occurred as a result of release from interspecific competition (Fig. 2). We note that $Z_{PC1}$ was a composite trait that reflects to a large degree the $lag_{ace}$ trait (while $Z_{PC2}$ reflects the maximum growth rate on the glucose trait, $r_{MAX\text{-}GLU}$). We determined the mean trait values (i.e. $Z_{PC1}$) for each derived ecotype in each derived population. We collected these data from all derived populations (plus the slow- and fast-switcher source ecotypes from each source population) into a single data set, and conducted principal components analysis (as above), again to generate composite trait descriptions that are independent (and orthogonal) in phenotype space. We conducted a hierarchical cluster analysis on the 126 ecotypes that resulted in nine clusters (Fig. 3). Examination of the identities revealed that clustered derived ecotypes were from different derived populations. Thus, parallelism was apparent in our experiment. Figure 4 illustrates the 'mean' growth curve of each cluster, compared with the growth curves of the progenitor slow-switcher and fast-switcher founding ecotypes. We mapped the nine clusters identified in Fig. 3 onto $Z_{PC1}$, and ranked them according to their mean $Z_{PC1}$ score (Fig. 5). We scored all the derived ecotypes using the rank of the cluster to which it belonged, and

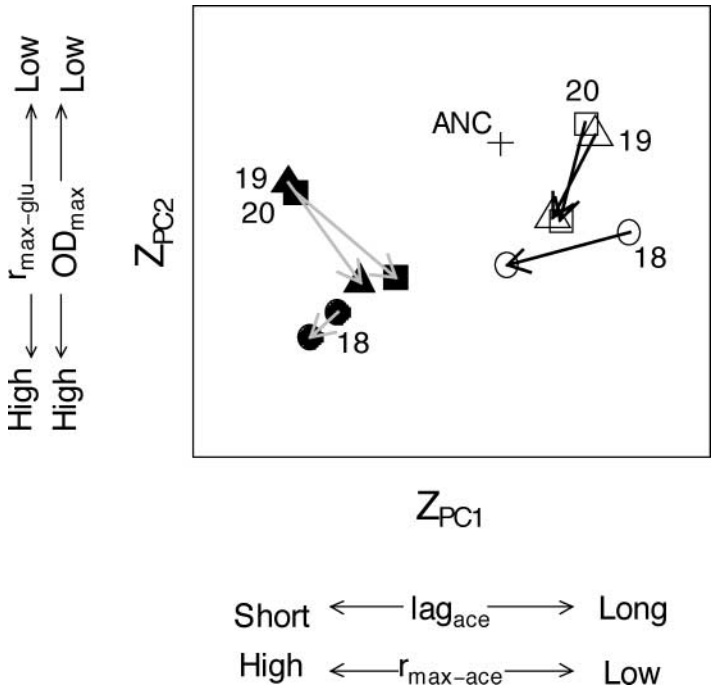


**Fig. 2.** Upon competitive release, mean phenotypes for slow-switcher (open symbols) and fast-switcher (filled symbols) populations generally converged in $Z_{PC1}$ and shifted down in $Z_{PC2}$. Arrows depict evolutionary trajectories of mean trait values, connecting source populations (generation 0, arrow tail), and derived populations (generation 200, arrowhead). ○, population 18; △, population 19; ▽, population 20. For reference, the ancestor (ANC) that gave rise to the source ecotypes used in this study is indicated with the +. For details, see Tyerman *et al.* (2008).

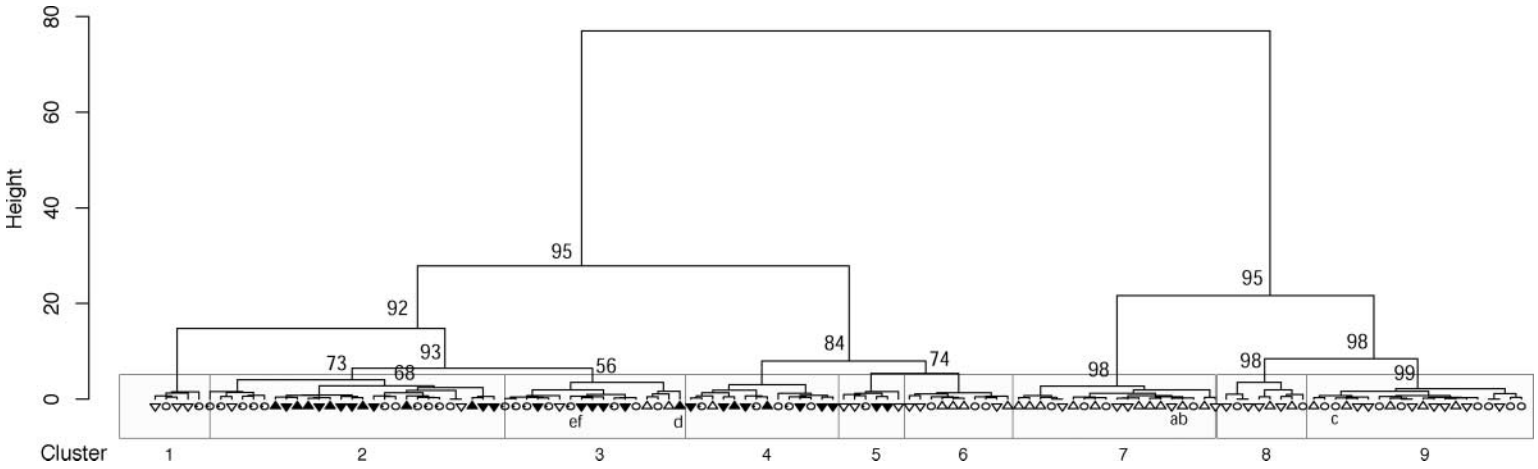


**Fig. 3.** Cluster analysis of 126 ecotypes from 30 derived populations and founding populations. Branch lengths (heights) represent the Euclidean distance in phenotype space. Derived ecotypes are labelled by shape, reflecting source population (○, population 18; △, population 19; ▽, population 20), and source ecotype (open symbols, slow-switcher; closed symbols, fast-switcher). Ancestral populations that served as the source ecotypes (slow- and fast-switchers) are indicated by letters: a, 19-SS; b, 20-SS; c, 18-SS; d, 18-FS; e, 19-FS; and f, 20-FS. Thin boxes outline clusters of 'convergent ecotypes' that are closer than 5 units in phenotype space. Numbers under the thin boxes denote cluster identity (see text). The numbers above selected branch nodes represent the bootstrap support for this cluster hypothesis.

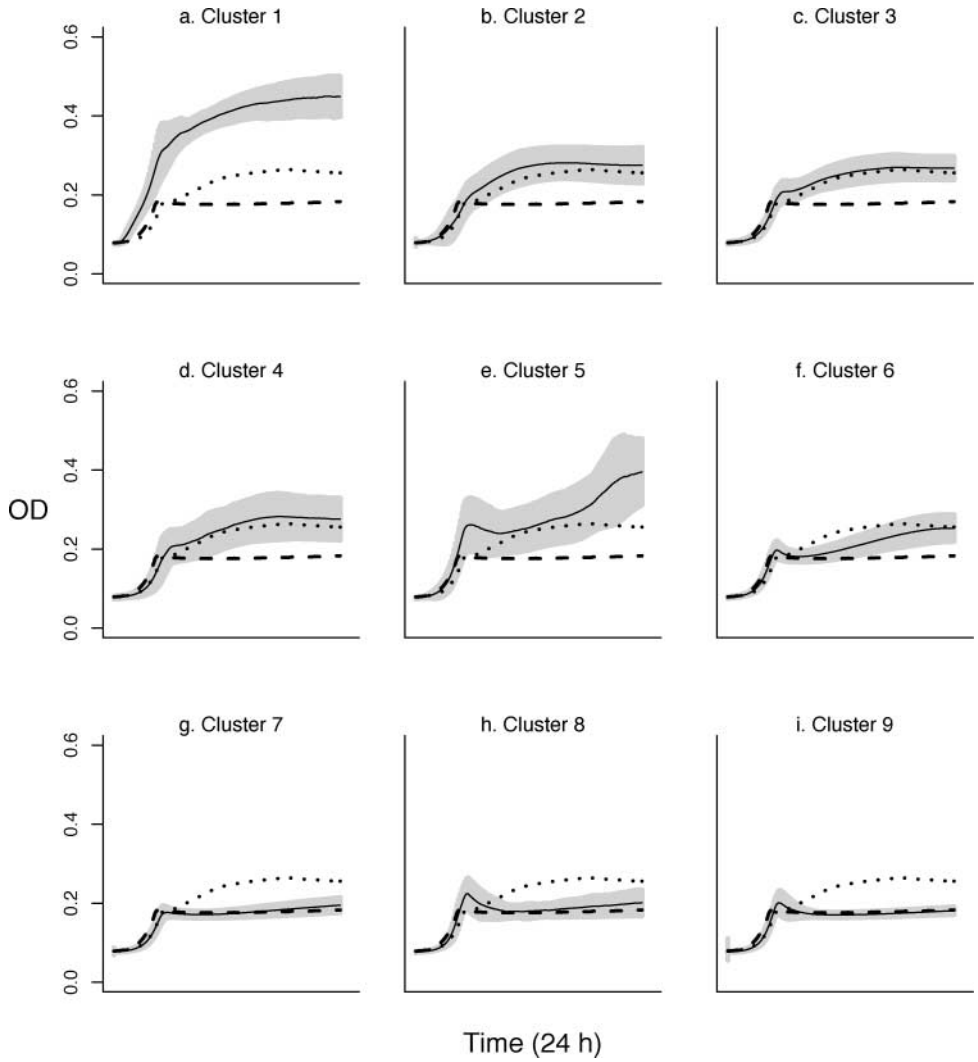


**Fig. 4.** Growth curves of derived ecotypes from the nine clusters (a–i) identified in the cluster analysis (Fig. 3). The x-axis (time) and y-axis (optical density, OD, 600 nm) is the same for each panel. The dashed and dotted lines are slow- and fast-switcher source ecotypes respectively (see Fig. 1), shown in each panel for comparison. The solid line illustrates the mean growth curve for that cluster, and the grey region denotes growth curve values within ± 1 standard deviation of the mean growth curve, calculated for each time point.

conducted the subsequent analyses using these ranks. Using ranks, we calculated the range of radiation as the range separating the maximum and minimum cluster rank for each replicate radiation. We then assessed whether the variation in range could be explained by source population or source ecotype (Table 2). We found that source ecotype ($F_{1,24} = 7.03$, $P = 0.014$) but not source population ($F_{2,24} = 2.40$, $P = 0.11$) explained variation in range of radiation, with slow-switcher derived populations accessing more phenotype space than

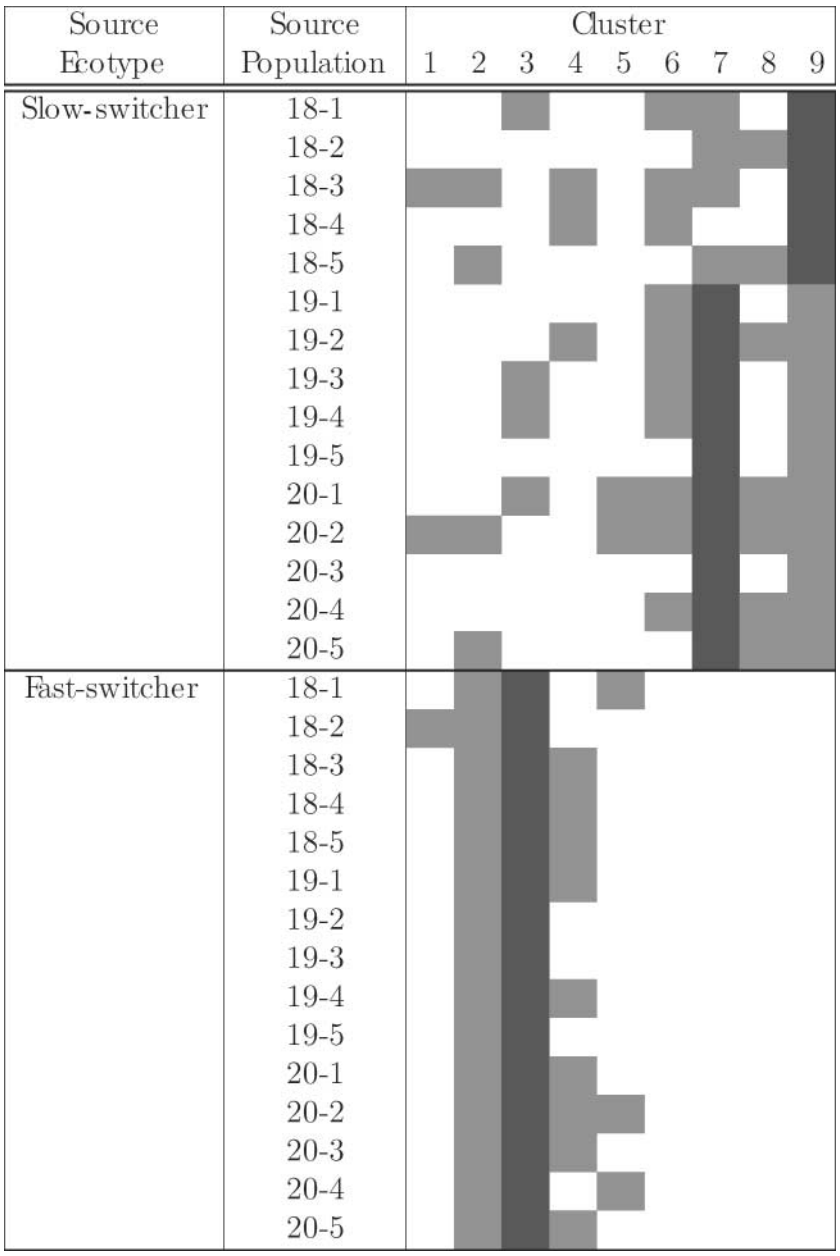


**Fig. 5.** Derived ecotypes isolated from founding populations (rows) were classified according to one of nine clusters (columns). Grey and white boxes indicate the presence and absence of a particular cluster respectively within each population. Dark grey boxes indicate the cluster of the ancestor that founded each population. The range of adaptive radiation was calculated as the range (in cluster-ranks) spanned by a derived population.

**Table 2.** Summary of ANOVA conducted on range of adaptive radiation (i.e. breadth of diversification along $Z_{PC1}$) after 200 generations of evolution under competitive release

| Source of variation | d.f. | SS | MS | $F$ | Pr(>$F$) |
|---|---|---|---|---|---|
| Population | 2 | 17.867 | 8.933 | 2.40 | 0.11 |
| Ecotype | 1 | 26.133 | 6.133 | 7.03 | **0.014** |
| Population × ecotype | 2 | 0.267 | 0.133 | 0.036 | 0.96 |
| Residuals | 24 | 89.200 | 3.717 | | |

fast-switcher derived populations (Fig. 5). (See Online Appendix for alternate analyses: evolutionary-ecology.com/data/2607appendix.pdf.)

## DISCUSSION

The propensity to diversify has the potential to determine the extent and form of diversity evolved under adaptive radiation. Here, using bacterial ecotypes isolated from experimental populations that had undergone adaptive radiation, we examined whether there were intrinsic differences between source ecotypes to undergo subsequent adaptive radiation. We found that slow- and fast-switcher *E. coli* ecotypes differed in their propensities to diversify, resulting in variation to the extent and form of variants derived under subsequent adaptive radiation. Populations derived from slow-switchers accessed more regions of metabolic phenotype space than populations derived from fast-switchers. As a consequence, slow-switchers diversified more than fast-switchers. These differences culminated in many slow-switcher populations evolving a fast-switcher ecotype within generations; however, fast-switcher populations never evolved the slow-switcher ecotype. Thus, the original adaptive radiation recurred only when initiated from populations founded by the slow-switcher, indicating that diversity in our experimental system is sensitive to the initial (genetic) conditions.

Our data support the contention that, under isogenic starting conditions, variation in mutational input can result in variation in patterns of diversity evolved under adaptive radiation, even when extrinsic factors – selection and ecological opportunity – are similar.

Other studies have observed differential ability to diversify among bacterial ecotypes (Spiers *et al.*, 2002; Buckling *et al.*, 2003; MacLean *et al.*, 2005; Spencer *et al.*, 2008). Using wrinkly-spreader ecotypes from a *Pseudomonas fluorescens* system, Spiers *et al.* (2002) compared the ability of various wrinkly-spreader$^-$ (i.e. 'smooth' revertants) to undergo subsequent diversification, compared with the ancestral ('smooth') ecotype. The authors observed variation among revertants in ability to diversify. Using the same experimental system, Buckling *et al.* (2003) evolved *Pseudomonas* in static broth microcosms, selecting the dominant genotype after each bout of diversification, and monitored the ability to diversify. The authors found that the dominant genotype had reduced ability to diversify as the experiment progressed and argued that adaptation limited the ability to diversify (but see Spencer *et al.*, 2008). Finally, MacLean *et al.* (2005) evolved *Pseudomonas*, initiated from mixtures of *pan* + and *pan* – isogenic lines. While the pan marker had no initial effect on fitness, the authors found that the degree of subsequent divergence under adaptive radiation was dependent on the initial marker state, suggesting an important role for intrinsic factors in adaptive radiation.

Our results extend these findings in two important ways. First, our results on *E. coli*, together with those of Spencer *et al.* (2008), extend the findings and observations on differential variation production beyond *Pseudomonas*. Second, our results can be placed against a well-characterized ecological context. In previous studies, we have developed a detailed understanding of the utility and functional consequences for the phenotypic variation evolved in our experimental populations (Friesen *et al.*, 2004; Tyerman *et al.*, 2005, 2008; Spencer *et al.*, 2007a, 2008; Le Gac *et al.*, 2008). In addition, because we have characterized the diversity that evolved in the original populations under adaptive radiation, we have an expectation for patterns of diversity expected under subsequent bouts of adaptive radiation in the same environment.

The current study complements two earlier studies by our research group. As noted above, we have previously characterized the mean phenotypic changes that occurred in these derived populations (Tyerman *et al.*, 2008). Those findings indicated that, during convergence due to release from competition for limited carbon resources, the slow-switcher derived populations 'closed the gap' to a much larger extent than did the fast-switcher derived populations. The findings of the present study reveal that slow-switcher derived populations simply contained more variants than did fast-switcher derived populations, with more variants oriented towards the fast-switcher region of phenotype space. The second study, by Spencer *et al.* (2008), characterized the propensity to diversify using 'fossil' populations of these strains prior to their evolutionary diversification. The authors found that the propensity to diversify increased as the fossil sample point approached the actual point of diversification, and attributed this result to a shifting adaptive landscape – due to frequency-dependent selection – that was becoming ever more permissive to invasion by the fast-switcher ecotype. Thus, Spencer *et al.* (2008) focused on the extrinsic factors that favour diversification in the ancestors that gave rise to both the slow- and fast-switcher ecotypes. The authors rejected a role for intrinsic factors ('genetic constraints') because proto-fast-switchers were occasionally produced by the pre-diversified fossil populations. Here, we start with full-fledged fast-switchers and argue that their inability to produce a slow-switcher is likely due to genetic constraints (see below).

The importance of intrinsic factors to explaining the origin and maintenance of phenotypic diversity may extend beyond our findings in the laboratory to microbes in nature, and to suites of phenotypic traits beyond metabolism (e.g. antibiotic resistance). Furthermore, these findings may be of relevance to the study of adaptive radiation in general; our results suggest that a greater emphasis be given to the generation of novel phenotypic variation when explaining patterns of evolved diversity.

## Caveats

Our interpretation of the data requires several caveats. First, in testing for variation in propensity to diversify, we required an experimental system that controlled for extrinsic factors (i.e. ecological opportunity and selection). Failing this, we could not argue that variation in propensity to diversify was due to variation in intrinsic factors *per se* (e.g. variation in propensity to diversify could be confounded by variation in opportunity or selection).

Because the vacant niches available to populations founded by different ecotypes were complementary, they were not exactly equivalent, and therefore could reflect differences in ecological opportunity. If the opportunity for a fast-switcher founded population to diversify to fill the niche previously occupied by the slow-switcher was in some way

diminished, then this reduced opportunity could result in the observed lower levels of diversity in fast-switcher derived populations. We offer three arguments against this line of reasoning. First, Tyerman *et al.* (2008) noted that the proportion of slow-switchers in the original diversified source populations was substantial (about 78%). Thus, it is not immediately apparent that the niche is fundamentally limited or intrinsically marginal. Second, from a physiological perspective, the hypothesized role filled by slow-switchers in this model adaptive radiation is as a fast-growth-on-glucose specialist, having reduced performance on acetate (Friesen *et al.*, 2004; Tyerman *et al.*, 2008). As acetate specialists, fast-switchers – having traded off growth performance on glucose for enhanced growth performance on acetate – still grow on glucose at the outset of diauxic growth (Tyerman *et al.*, 2008). Thus it is not logically consistent that the ample glucose made available in the daily batch environment – which is consumed by the fast-switchers – should be considered as negligible or absent as an opportunity by the fast-switcher ecotype. Third, Spencer *et al.* (2008) showed, in the evolution of the diversified populations prior to diversification, there was evolution towards the slow-switcher ecotype. We interpret this to indicate that the ancestor had been selected to evolve towards being a slow-switcher and to fill the opportunity of the slow-switcher niche. For these reasons, we feel that the reduced diversity seen in the fast-switcher derived populations was not a direct result of reduced ecological opportunity.

A second criticism of our interpretation of the results is that the nature of selection acting on slow- and fast-switcher populations may have differed. To understand this criticism, it helps to first consider our data in light of theory developed for sympatric ecological speciation (Dieckmann and Doebeli, 1999; Doebeli and Dieckmann, 2003). Based on adaptive dynamics theory (Geritz *et al.*, 1998), competition for resources can generate evolutionary dynamics characterized by two phases. In the first phase, directional selection causes a population to evolve towards an evolutionary branching point in phenotype space. Upon occupying the evolutionary branching point, negative frequency-dependent selection leads to a shift in the nature of selection. Here, the selection regime turns from directional to disruptive, and causes the population to undergo evolutionary branching and to diverge into two phenotypic clusters, which can be illustrated using pairwise invasibility plots (Geritz *et al.*, 1998), as shown in Fig. 6. Note that according to the evolutionary dynamics resulting from the situation shown in Fig. 6, evolutionary branching and diversification should occur irrespective of the starting phenotype. In particular, evolutionary branching should occur even if the starting phenotype is a fast-switcher, contrary to our observations. If we assume that the invasion conditions outlined in the evolutionary model presented in Fig. 6 are indeed met in our experimental system (Friesen *et al.*, 2004), we conclude that our results diverge from the predictions of the model because of mutational constraints in populations initiated by fast-switchers. However, instead of the differences in variational constraint facing slow- and fast-switcher derived populations, one could also envisage that the conditions for invasion and evolutionary branching do not hold for populations initiated by fast-switcher ecotypes. This could happen if the pairwise invasibility plot looks different from the one shown in Fig. 6. For example, Fig. 7 shows a more complicated pairwise invasibility plot, according to which evolutionary branching and diversification would still occur when the starting phenotype is a slow-switcher, but would not occur, or would be less likely to occur, with a fast-switcher as starting phenotype. This is because according to the pairwise invasibility plot shown in Fig. 7, an initial population of fast-switchers would not evolve to the same branching point as a population of slow-switchers, and instead would evolve to a local ESS consisting of fast-switchers that cannot be invaded by nearby

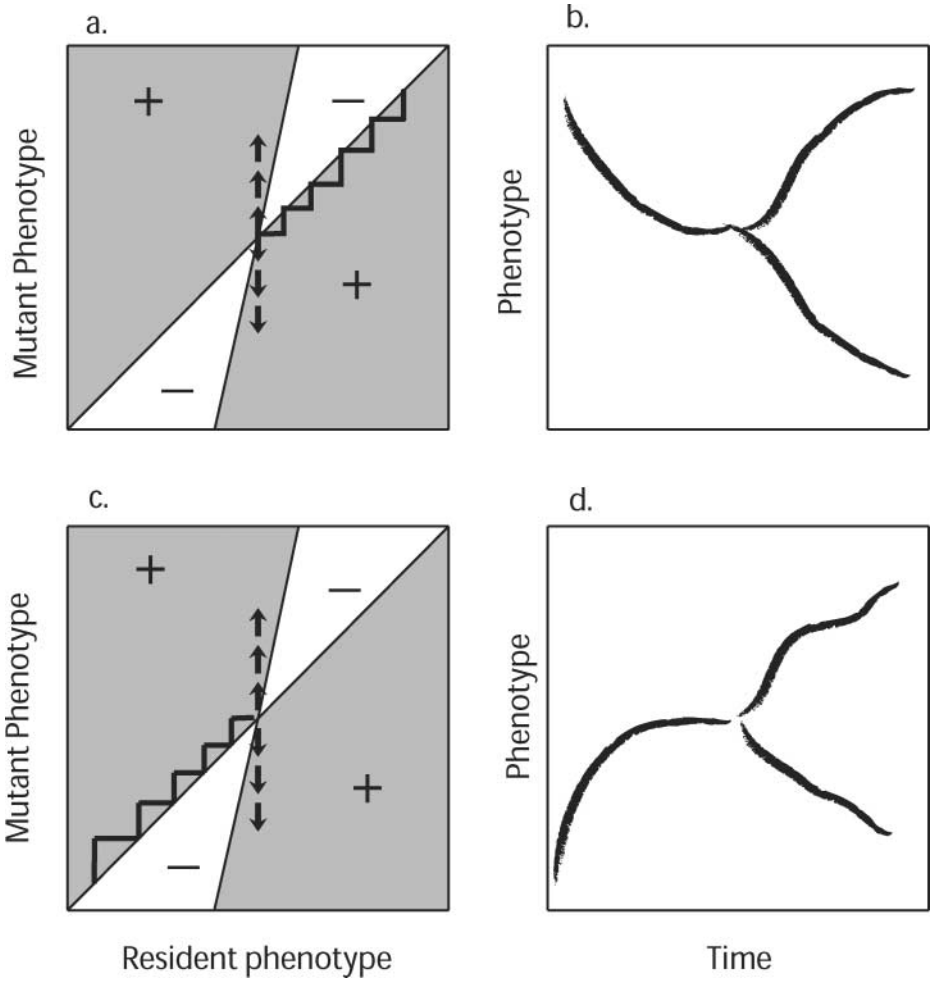


**Fig. 6.** Pairwise invasibility plots (a and c) and corresponding evolutionary trajectories (b and d) illustrating scenarios of adaptive diversification. The pairwise invasibility plots show regions of positive invasion fitness (grey) and negative invasion fitness (white) for all combinations of resident (x-axis) and mutant phenotype (y-axis). To derive the evolutionary trajectory, it is assumed that if a mutant can invade a resident population (i.e. if the corresponding resident–mutant pair lies in the grey region), then the mutant replaces the resident and becomes the new resident. This substitution is indicated by the thick black line segments in (a) and (c). Note that along the diagonal, mutant phenotypes are equal to resident phenotypes, and hence invasion fitness is 0 (i.e. mutant and resident have the same fitness). Evolutionary equilibrium points, or evolutionary singularities, are given as intersection points of the diagonal with the 0-isocline of the invasion fitness function (Geritz *et al.*, 1998). The slope of this 0-isocline at the evolutionary singularity determines the evolutionary dynamics. In the situation shown here, the singularity is an evolutionary branching point: irrespective of the starting phenotype the resident population first evolves towards the singularity, which is therefore an evolutionary attractor. At the branching point, selection becomes disruptive due to negative frequency dependence. As a consequence, every nearby mutant can invade, and hence the population diversifies into two separate phenotypic clusters (black arrows in a and c), resulting in the evolutionary trajectories shown in (b) and (d).

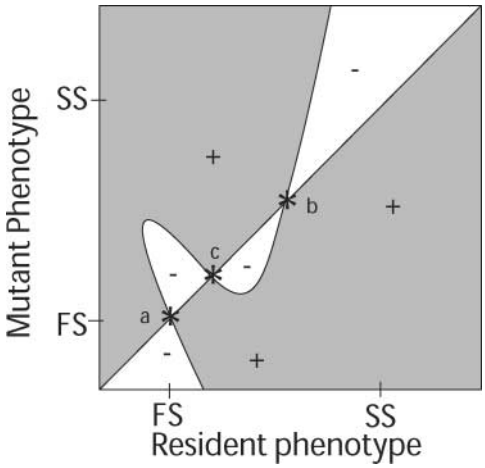


**Fig. 7.** Hypothetical pairwise invasibility plot showing multiple equilibria (i.e. multiple intersection points) between the 0-isocline of the invasion fitness and the diagonal (*). In this case, the evolutionary dynamics depend on initial conditions. Populations initiated from slow-switchers (SS) would evolve to the branching point (b) and diversify as in Fig. 6. However, populations initiated from fast-switchers (FS; i.e. with small phenotypic values on the x-axis) would evolve towards a local evolutionarily stable strategy (ESS; a), i.e. to a phenotype that is non-invadable by nearby mutants. In this hypothetical example, the branching point corresponding to slow-switchers and the ESS corresponding to fast-switchers are separated by an evolutionary repellor (c), the existence of which leads to dependence of the evolutionary dynamics on initial conditions.

mutations. Effectively, due to the more complicated nature of the pairwise invasibility plot in Fig. 7, the corresponding evolutionary dynamics has more than one possible equilibrium point.

Thus, selection of the type reflected in Fig. 7, rather than mutational constraint, could account for asymmetries in divergence. Our response to this criticism is twofold. First, while we can imagine complex ecological scenarios involving, for example, combinations of competition and facilitation leading to diversification (Friesen *et al.*, 2004; Tyerman *et al.*, 2005; Saxer *et al.*, 2009), we have no evidence that such complexity is operating in our system (i.e. whereby intermediate levels of slow-switching have negative invasion fitness, yet extreme levels of slow-switching have positive invasion fitness). We acknowledge that complex invasion scenarios are possible, and thus we remain open to a role for extrinsic factors in explaining our data. Second, in contrast to the possibility of selection against slow-switchers in resident populations of fast-switchers, competition experiments have confirmed our belief that there is strong selection for slow-switchers to invade resident populations of fast-switchers. We have previously shown that, when rare, slow-switcher ecotypes can invade populations of fast-switchers (Friesen *et al.*, 2004; Tyerman *et al.*, 2005).

Most crucial for our contention that mutational constraint, and not selection, was responsible for the asymmetry in diversification was a competition experiment we conducted between intermediate slow-switcher and intermediate fast-switcher ecotypes. We initiated these mixed populations to assess whether interspecific competition could drive ecological character divergence (for further details. see Tyerman *et al.*, 2008). Prior to divergence between the lineages descended from the intermediate slow-switcher and intermediate

fast-switcher, we noted that the intermediate slow-switcher lineage initially increased in frequency (in ten of ten replicates). This increase occurred during the first 24 h of competition, before the generation of novel, divergent phenotypes. We interpret these results as selection favouring the intermediate slow-switcher ecotype when competed against the fast-switcher ecotype. These empirical measures of selection from competition experiments indicate that there is selection for slow-switcher (or intermediate slow-switcher) phenotypes in fast-switcher populations, and thus the absence of slow-switchers in fast-switcher derived populations is unlikely to result from selection against slow-switchers.

## Possible mechanisms

We have not identified the genetic mechanisms that lead to variation in propensity to diversify in our experimental populations. In particular, we are curious about the apparent genetic constraints that limit fast-switchers from subsequent metabolic diversification. A previous study involving analogous slow- and fast-switcher *E. coli* ecotypes identified an IS5 insertion that disrupted *iclR* function (Spencer *et al.*, 2007a). This mutation caused the constitutive expression of acetate-metabolizing genes (i.e. *aceBAK*), although other mutations leading to similar fast-switcher phenotypes were evident. We suspect that similar genetic mechanisms are in operation in the experimental populations used in this study. That is, mutations that target regulatory systems responsible for catabolite repression may be involved in the genetic changes leading from the ancestral *E. coli* (Le Gac *et al.*, 2008; Spencer *et al.*, 2008; Tyerman *et al.*, 2008) or slow-switcher ecotype (this study) to the fast-switcher ecotype. Indeed, there is now evidence that at least some fast-switchers carry a mutation in the *arcA* gene that affects the activity of this important regulator of glucose and acetate pathways (M. Le Gac and M. Doebeli, unpublished data). Mutations that disrupt function via deletion, inversion or point-mutation may not be easily reversed to reconstitute the original phenotypic function (although compensatory mutations may aid in reconstitution). If the probability of disrupting regulation is greater than the probability of reconstituting regulatory function, then we might expect variation in the propensity to diversify to be common.

'Deregulation' (reviewed by Kassen and Rainey, 2004) has often been identified in studies focused on the genetic mechanisms underlying diversification in other model adaptive radiations, including the wrinkley-spreader morphs in *Pseudomonas fluorescens*, the GASP forms in *E. coli* (Finkel and Kolter, 1999; Zinser and Kolter, 2004), and cross-feeding ecotypes in *E. coli* (Rosenzweig *et al.*, 1994; Treves *et al.*, 1998). We speculate that deregulation may often be involved in the generation of novel phenotypes in adaptive radiation with microbes. Deregulation leading to constitutive expression is a clear example of genetic assimilation. Genetic assimilation is the evolutionary reduction of trait sensitivity to an environmental stimulus (Waddington, 1953; West-Eberhard, 2003). In the case of catabolite repression, the ancestor and slow-switcher ecotypes are highly sensitive to the glucose environment, corresponding to the regulated inhibition of acetate-metabolizing genes. Through genetic assimilation, the fast-switcher may have evolved reduced sensitivity to the glucose environment, via mutations that disrupted the regulation associated with catabolite repression (Spencer *et al.*, 2007a). If true, our findings may be of relevance to medically important strains. Recently, Hoboth *et al.* (2009) found evidence for evolution towards constitutive expression (i.e. genetic assimilation) of metabolic traits in highly mutable strains of *Pseudomonas aeruginosa* isolated from patients with chronic lung disease associated with cystic fibrosis.

The ecological theory of adaptive radiation pertains to the external factors that drive

phenotypic and ecological diversification. Internal factors, such as the mutational bias suspected in this study, provide additional information about constraints that may be operating in the system. While often under-appreciated, these constraints can explain divergent outcomes under adaptive radiation. Our study provides greater motivation to include internal factors in a more general theory of adaptive radiation.

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