Evolutionary history and genetic parallelism affect correlated responses to evolution

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Abstract:

We investigated the relationship between genomic and phenotypic evolution among replicate populations of _Escherichia coli_ evolved for 1000 generations in four different environments. By resequencing evolved genomes, we identified parallel changes in genes encoding transcription regulators within and between environments. Depending on both the environment and the altered gene, genetic parallelism at the gene level involved mutations that affected identical codons, protein domains or were widely distributed across the gene. Evolved clones were characterized by parallel phenotypic changes in their respective evolution environments but also in the three alternative environments. Phenotypic parallelism was high for clones that evolved in the same environment, even in the absence of genetic parallelism. By contrast, clones that evolved in different environments revealed a higher parallelism in correlated responses when they shared mutated genes. Altogether, this work shows that after an environmental change or the colonization of a new habitat, similar ecological performance might be expected from individuals that share mutated genes or that experienced similar past selective pressures.

Keywords : adaptation ; Epistasis ; experimental evolution ; genome sequencing ; parallelism
Introduction

Next-generation sequencing (NGS) technologies are revolutionizing our understanding of the genetics of adaptation. From evolution studies focusing on specific candidate genes, investigations have now switched to the genome scale. Applied to experimental evolution of microorganisms, genome comparisons of ancestral and evolved clones enable the identification of virtually all the mutations associated with experimental adaptation (reviewed in Brockhurst et al. 2011; Conrad et al. 2011; Dettman et al. 2012; Hindré et al. 2012). A prominent finding has been that adaptation of independent replicate populations to a given environment often involves genetic parallelism, characterized by mutations that affect identical genes, operons, or functional operational units (Herring et al. 2006; Barrick et al. 2009; Conrad et al. 2009; Tenaillon et al. 2012). However, the precise mutations altering these genetic loci are often different in replicate bacterial populations (Woods et al. 2006; Tenaillon et al. 2012).

Consideration of the genes that are repeatedly mutated during evolution experiments finds that many are global regulators of gene expression, highlighting the involvement of regulatory network rewiring in adaptation (Philippe et al. 2007; Wang et al. 2010; Conrad et al. 2011; Hindré et al. 2012). The same global regulator-encoding genes are often mutated in distinct experimental settings indicating that identical regulatory hubs may provide adaptation to contrasting environments (Conrad et al. 2011). Moreover, non-additive, i.e. epistatic, interactions between mutations have been shown to be of primary importance in explaining both the evolutionary trajectory of a population and the phenotypes of evolved individuals (Bull et al. 2000; Remold & Lenski 2004; Weinreich et al. 2005; 2006; Poelwijk et al. 2007; Cooper et al. 2008; Almahmoud et al. 2009; Le Gac & Doebeli 2010). Beneficial mutations in global regulator-encoding genes likely reorganize the architecture of the cellular regulatory networks, potentially contributing to the influence of epistasis (Khan et al. 2011; Tenaillon et
al. 2012), and to complex phenotypic changes in environmental conditions distinct from the evolution environment (i.e., correlated responses).

In the present study, we investigated the effect of beneficial mutations on correlated responses to evolution. Replicate populations evolving in identical environments are often characterized by having high phenotypic parallelism. The extent to which this parallelism applies to correlated phenotypic responses is, however, less well studied. At one extreme, replicate populations may display parallel correlated responses in many alternative environments. At the other, they may display varied phenotypes under alternative conditions and phenotypic parallelism only in the evolution environment. These phenotypic outcomes have important ecological implications since they dictate how ecologically similar populations will cope with environmental challenges such as the colonization of new habitats/hosts or the modification of ecosystems at both the biotic and abiotic levels. Only few studies using model bacteria have investigated the correlated phenotypic responses to evolution, including some that revealed parallel losses of catabolic abilities and growth phenotypes on alternative resources (Cooper & Lenski 2000; Barrett et al. 2005; Fong et al. 2005). However, in other studies using the same bacterial species, populations have been found to be more variable when assayed for growth on alternative resources than on the resource on which they first evolved (Travisano et al. 1995; Travisano & Lenski 1996; MacLean & Bell 2003; Ostrowski et al. 2005; 2008). The genetic changes associated with adaptation to a given evolution environment have only rarely been investigated for their effects on correlated responses in alternative environments. Intuitively, one may expect the correlated responses to be more similar for clones sharing mutated genes, but the relationship between genetic parallelism and phenotypic performance in alternative environments may be complex. For example, different mutations affecting a given gene may have similar phenotypic effect in the evolution environment but not necessarily in another one, especially in the case of global regulator-encoding genes.
Conversely, even if replicate populations adapt through mutations in different genes, the phenotypic consequences of these mutations could be similar not only in the evolution environment, but also under alternative environmental conditions. Here, we propagated four replicate populations of *Escherichia coli* for 1000 generations in each of four different environments. We sequenced the genome of one evolved clone isolated from each of the 16 populations, allowing us to identify mutations associated with evolution in each environment. We assayed the phenotype of isolated clones in their evolution environment as well as in the three alternative ones to provide a measure of the direct and correlated phenotypic responses to evolution in all four environments. Using this experimental design, we investigated first, the level of genetic parallelism associated with evolution in several environments and second, the respective effect of genetic parallelism and evolutionary history on the correlated responses to evolution. In particular, we tested whether phenotypes in alternative environments (correlated responses to evolution) were more similar for clones sharing mutations in identical genes or genetic loci (genetic parallelism) irrespective of the environment in which they evolved (evolutionary history) or for clones that evolved in the same environment irrespective of genetic parallelism.

**Material and methods**

*Experimental evolution*

*E. coli* B REL606 (Lederberg 1966; Jeong *et al.* 2009) was used as the ancestor to found four replicate populations that were propagated by daily serial transfer in each of four different environments for 1000 generations at 37 °C. Population samples were collected at 100-generation intervals and frozen at -80 °C as glycerol suspensions in the same conditions as the ancestor. All four environments are based on Davis minimal (DM) medium (Lenski *et al.* 1991). The first environment, named Ace, comprised 15 mL DM supplemented with 2 g/L
sodium acetate trihydrate in 50-mL flasks shaken at 200 rpm. The second, named Gly, comprised 15 mL DM supplemented with 1 g/L glycerol in static Petri dishes. The third, named Glc, comprised 15 mL DM supplemented with 1 g/L D-gluconate in test tubes shaken at 200 rpm. The fourth, named Glu, comprised 600 µL DM supplemented with 1 g/L D-glucose in 1 mL x 96-well plates shaken at 200 rpm. These four environments were chosen in order to apply a combination of parameters impacting bacterial growth and adaptation due to contrasting carbon sources, and homogenization and oxygenation levels. This choice was made \textit{a priori} to allow a wide range of mutational targets, including genes associated with metabolic pathways, such as the catabolism of a specific carbon source, but also regulatory genes that influence a combination of growth aspects such as carbon source consumption and better oxygen use. This strategy should also favor the selection of mutations with large correlated phenotypic effects in alternative environments. Every day (24 +/-2 hours), populations were diluted 300-fold into the same fresh medium, allowing \( \sim 8.2 \log_{2} (300) \) generations per day. Populations therefore experience every day a lag phase with no detectable growth, followed by an exponential phase characterized by the optimal consumption of the available carbon source, and finally a stationary phase when the carbon source has been exhausted (except in the Ace environment, see below), before being diluted again in fresh medium. The bottleneck at each daily transfer was never fewer than \( \sim 4 \times 10^6 \) cells in any environment. After 1000 generations, each of the 16 populations was streaked on LB agar plates that were incubated overnight at 37 °C. A single colony was randomly chosen from each plate and frozen at -80 °C as a glycerol suspension.

\textit{Growth profile assays}

Growth profiles were measured first for population samples collected every 200 generations, in triplicate and in their respective evolution environments, and second for the ancestor and
individual evolved clones with five-fold replication and in the four different environments.

For each experiment, a physiological pre-acclimation was performed in the assay environment, consisting of an overnight culture of bacterial cells followed by a 300-fold dilution and a 24-h incubation. This pre-acclimation step differed slightly for the growth profiles in the Ace environment, in which populations that evolved in the three other environments, as well as the ancestor, grew slowly and were still in exponential phase after 24 hours of incubation. In this particular case, the initial overnight cultures were performed in Glu. For all experiments and after a 300-fold dilution, pre-acclimated cultures were incubated in the relevant assay environment.

Growth profiles were determined by measuring the optical density for each culture at 600 nm (OD$_{600}$) at regular intervals during 24 h of incubation. We used the resulting growth curves to calculate the maximum growth rate ($\mu_{\text{max}}$) of each evolved culture (populations and individual clones) relative to the ancestor. For the Gly, Glc and Glu environments, maximum growth rates were measured between 0.2 and 0.8 of the maximal ancestral OD$_{600}$. In the Ace environment, the ancestor was still in exponential phase after 24 h of culture, and growth rates were measured between 0.5 and 1 times the OD$_{600}$ reached by the ancestor following 24 h of growth.

Fitness assays

Fitness assays were performed for the populations sampled at 300, 600 and 1000 generations in their respective evolution environments, and for the individual evolved clones in all four environments. All assays were replicated five-fold. competitions were performed as previously described (Lenski et al. 1991). Briefly, all competitors including the REL606 ancestor and a marked phenotypic variant called REL607 were pre-acclimated in the assay environment. The ancestor REL606 and all derived evolved clones are unable to use
arabinose as a carbon source (Ara⁻), while REL607 is a REL606 spontaneous revertant that recovered this catabolic ability (Ara⁺). After pre-acclimation, each evolved sample (population and individual clones) and the REL606 ancestral strain as a control were mixed separately with REL607 at a 1:1 ratio. Mixtures were then diluted 300-fold in fresh medium and incubated for 24 h at 37 °C in the assay environment. At days 0 (when the two competitors are mixed) and 1 (after 24 h of incubation) of each competition experiment, cells were diluted and plated on indicator tetrazolium arabinose (TA) plates, on which Ara⁺ or Ara⁻ colonies appear pink or red, respectively (Lenski et al. 1991). Plates were incubated 24 h at 37 °C and each of the competitors was scored. Using the initial and final cell counts we calculated the realized (net) population growth of each competitor, according to the following formula: \( \frac{G_i = \ln(C_{t1} \times 300/C_{t0})}{C_{t0}} \), where \( C_{t0} \) and \( C_{t1} \) are the number of colonies at the beginning and after 24 hours of competition, respectively, and 300 the 300-fold higher dilution factor required for \( C_{t1} \) compared to \( C_{t0} \). The fitness of one competitor relative to the other was then calculated as the ratio of their net growth rates during the competition experiment according to the formula: \( \text{Fitness} = \frac{G_{Ara^-}}{G_{Ara^+}} \), where \( G_{Ara^-} \) and \( G_{Ara^+} \) are the realized population growth of the Ara⁻ ancestor and evolved clones and of the Ara⁺ REL607 clone, respectively (Lenski et al. 1991).

Genome sequencing

The genome of each of the 16 clones isolated after 1000 generations of evolution was re-sequenced on the Illumina HiSeq2000 platform (GATC Biotech, Germany) using one lane of single-end 35-bp reads. Barcodes were used for each genome so that reads were clone-specific. Candidate point mutations were identified in comparison to the ancestral genome of REL606 (Jeong et al. 2009) using the SNiPer pipeline (Wielgoss et al. 2011). The existence of a mutational event was inferred when it was identified in more than 75% of the reads.
covering a given site and in more than 20 reads. We identified a total of 54 mutations of which 25 were randomly chosen and confirmed by PCR and sequencing of the obtained products. Short read single-end re-sequencing reliably detects SNPs but may miss large indels and mutational events associated with mobile genetic elements (recombination and transposition). To minimize this potential bias, we checked the eight genes that had a mutation in more than one clone, i.e. mutated in parallel, for gene size polymorphism by PCR amplification. Using this approach, we identified two additional mutations in the \textit{glpR} gene: one IS1 insertion 30 bp upstream of its start codon and one 125-bp deletion in its coding region. Re-sequencing the genome of one single evolved clone sampled from each population allowed us to directly link phenotypic changes in different environments to specific combinations of mutations. This approach is not appropriate for analyzing genomic evolution at the level of the entire populations which is beyond the scope of our study and would imply re-sequencing genomes from either multiple evolved clones or mixtures of populations (Barrick & Lenski 2009; Herron & Doebeli 2013).

\textit{Statistical analyses}

As the same global regulator-encoding genes were mutated in more than one environment, we used a generalized linear model (GLM) to test if the correlated responses were more similar for clones that shared mutated genes and/or that evolved in the same environments. To build the model, we measured the variability of the phenotypes (average $\mu_{\text{max}}$ and fitness values) between clones in their alternative environments. For pairs of clones that evolved in the same environment we considered the three alternative environments, while for those that evolved in two different environments we considered the two shared alternative ones. The variability of the correlated responses was calculated as the mean squares among clones (MSS), as for an ANOVA, but we did not compare the MSS to the mean squares error (MSE) using F statistics.
Instead, MSS were ranked within each alternative environment and used as an ordinal response variable in the GLM. By doing this, we could consider as the units of interest the pairs of clones instead of the clones themselves, allowing for the choice of two explanatory variables. The first distinguished the pairs of clones sharing or not a mutated gene (nominal explanatory variable) and the second the pairs of clones that evolved or not in the same environment (nominal explanatory variable). Details of the input data used for the GLM are given in a Dryad file (doi:10.5061/dryad.n2582). Likelihood ratio $\chi^2$ values were used to test for significance of ordinal logistic regressions as implemented in JMP version 3.1.5 (SAS Institute).

Results

Phenotypic changes in the evolution environments

Four replicate populations of *E. coli* B were propagated for 1000 generations in each of four evolution environments that differed in carbon source, oxygenation and degree of spatial structure. We quantified the phenotypic evolution of each population in its respective evolution environment by measuring its maximum growth rate relative to the ancestor at 200-generation intervals ($\mu_{\text{max}}$; Fig. 1a), and its fitness relative to the ancestor at 300, 600 and 1000 generations (Fig. 1b). Compared to their common ancestor all populations increased in fitness and all except those evolved in Glu increased their $\mu_{\text{max}}$ after 1000 generations, indicating that they adapted to their environments (Table S1, Supporting information, Fig. 1). After 1000 generations of evolution, we isolated one evolved clone from each replicate population and measured $\mu_{\text{max}}$ and fitness in its evolution environment (Table S2, Supporting information, Fig. 2). Phenotypic evolution of the clones and their source populations was correlated ($\mu_{\text{max}}$: Spearman Rho = 0.93, $p < 0.0001$; fitness: Spearman Rho = 0.60, $p = 0.0144$). However, the clones isolated from the Glu environment tended to display a higher
than their entire source populations, indicating that they probably belong to specific sub-
lineages that may not be representative of the entire populations. This has however no impact
on our results since our main goal here is to relate phenotypes to specific combinations of
mutations, a task that could not be achieved by focusing on entire populations. Therefore, all
the following analyses focus on comparisons between phenotypes and genomes of clones and
*not* populations.

Clones sampled from the four environments had quantitative differences in their divergence
from the ancestor (Kruskal–Wallis one-way analysis of variance: $\mu_{\text{max}}, \chi^2 = 12.71, p = 0.0053$;
fitness, $\chi^2 = 11.59, p = 0.0089$), indicating different magnitudes of phenotypic evolution in the
four environments (Table S2, Supporting information, Fig. 2).

Genome sequencing

Re-sequencing the genomes of the 16 evolved clones revealed a total of 54 mutations
compared to the ancestor, with one to six mutations for each clone. These mutations affected a
total of 25 genes and included 53 SNPs and one 1-bp insertion (Table 1). Most SNPs (51)
occurred within genes, including 49 non-synonymous and only 2 synonymous changes. Only
three mutations affected intergenic regions, including two SNPs and the 1-bp insertion. Two
additional indels were subsequently identified (see Material and methods), one IS1 insertion
and one 125-bp deletion, both affecting the *glpR* gene (Table 1).

Genetic parallelism within and between evolution environments

Defining genetic parallelism as a mutational change occurring in identical genes, operons or
functional units (Tenaillon *et al.* 2012) in at least two clones, ~60 % (34/56) of the identified
mutations occurred in parallel (Fig. 3, Table 1). The proportion of parallel to total mutations
was 7/12, 13/19, 9/16, and 6/9 in the Ace, Gly, Glc, and Glu environments, respectively. This
parallelism involved eight genes or operons (Fig. 3, Table 1), including four specific to a
single environment (mreBC in Ace, glpR and glpK in Gly, and lldR in Glc), and four across
multiple environments (argR, spoT, rho, and nadR). In all cases genes that changed in parallel
within a single environment reflected a greater degree of environmental clustering than
expected if the mutations were distributed randomly over the evolved clones (Fisher’s exact
test, for all genes mutated in parallel \( p \leq 0.05 \)). These data are consistent with the changes in
these genes conferring environment-specific adaptations.

The four evolved clones sampled from the Ace environment had a total of 12 mutations
affecting nine different genes that can be grouped into eight loci. Three of these loci were
targeted by mutations in more than one clone: the mreBC genetic locus, mreB and mreC being
part of the same operon (Wachi et al. 2006), had mutations in two clones, while rho and argR
were mutated in three and two clones, respectively (Fig. 3, Table 1). The four clones sampled
from the Gly environment had a total of 19 mutations affecting ten different genes. Seven
mutations occurred in glpR and glpK, which were mutated in four and three clones,
respectively, while six occurred in spoT and rho, which were mutated in four and two clones,
respectively (Fig. 3, Table 1). The four evolved clones sampled from the Glc environment had
a total of 16 mutations affecting ten different genes, among which three (argR, lldR and spoT)
had mutations in three clones (Fig. 3, Table 1). Finally, the four evolved clones sampled from
the Glu environment had 9 mutations affecting a total of 5 different genes including spoT and
nadR that were mutated in four and two clones, respectively (Fig. 3, Table 1). Despite the
high overall level of genetic parallelism, the number of parallel changes found in individual
clones was quite variable (Fig. 3, Table 1), ranging from one (Ace_4, Glc_2 and Glu_2) to
four (Gly_3, Glc_4, Glu_1 and Glu_4).
Four genes were mutated repeatedly in different environments—*argR* (Ace and Glc environments), *spoT* (Gly, Glc and Glu environments), *rho* (Ace and Gly environments), and *nadR* (Gly and Glu). Therefore, evolved clones from all pairs of environments, except Ace/Glu, shared mutated genes, but the combinations of shared mutated genes were environment-specific (Fig. 3, Table 1). The highest level of genetic parallelism was detected for *spoT*, which was modified in 3/4 environments and 11/16 clones, further emphasizing previous observations of repeated *spoT* mutations (see below; Cooper *et al.* 2003; Cooper 2007; Ostrowski *et al.* 2008; Woods *et al.* 2011).

*Functions of mutated genes and distribution of mutations within repeatedly modified genes*

We investigated the function of the mutated genes by considering their Gene Ontology categories (Riley *et al.* 2006). Seven (*rpoA*, *rho*, *argR*, *lldR*, *glpR*, *flhD*, and *nadR*) of the 25 mutated genes are categorized as transcription regulators, which is more than expected by chance alone (Fisher one tail exact test $p = 0.01$). Although not classified in the GO transcription category, other mutated genes are also involved in transcription regulation: *fabR* encodes a transcription regulator, and *relA* and *spoT* are involved in the metabolism of ppGpp, which reprograms the entire transcription machinery in bacterial cells (Srivatsan & Wang 2008). Therefore, 10 of the 25 genetic loci that were modified during evolution are involved in transcription regulation. Strikingly, six of them (*rho*, *argR*, *lldR*, *glpR*, *nadR*, and *spoT*) are among the eight that were mutated in parallel, emphasizing the importance of changes in transcription regulation for adaptation.

Two sub-groups can be distinguished among these six genes. The first comprises *glpR* and *lldR*, which are local transcription regulators (Zeng *et al.* 1996; Aguilera *et al.* 2008) affecting the expression of a small number of genes involved in the consumption of a single carbon source (glycerol or lactate, respectively). Parallel changes occurred in these two genes only...
within specific environments (Gly or Glc, respectively). The second sub-group comprises
argR, which regulates a large set of target genes (Caldara et al. 2006), spoT and rho, which
are involved in global gene regulation (Srivatsan & Wang 2008; Epshtein et al. 2010), and
nadR, which regulates global cell physiology by integrating multiple environmental signals to
control the synthesis of NAD, a central co-factor in many bacterial metabolic pathways
(Grose et al. 2005). Interestingly, it is precisely this second group of transcription regulator-
encoding genes that had parallel changes between, as well as within, environments.
The genetic parallelism observed at the gene level was associated with mutations affecting
different codons largely distributed within these genes, with however two notable exceptions
(Table 1). First, specific regions of the same genes were affected by independent mutations,
including the 5’ end of argR and glpK. Second, nearby codons were affected by independent
non-synonymous mutations in rho (codons 322 and 324), and spoT (codons 207 and 209). In
addition, the exact same codons of rho (codons 322 and 324) and spoT (codon 393) were
repeatedly affected by independent mutations, leading to either the same or different amino-
acid changes in the corresponding proteins (Table 1). For both exceptions, similar mutations
occurred in more than one environment.

Correlated phenotypic responses to evolution in the alternative environments
Next, we investigated how adaptation to a given evolution environment affected \( \mu_{\text{max}} \) and
fitness in alternative conditions. The correlated response to selection of each of the 16 evolved
clones was compared to the common ancestor in each of the three environments in which it
did not evolve (hereafter called alternative environments). As an example, the four evolved
clones sampled from the populations that were propagated in Ace were assayed in the
alternative environments Gly, Glc and Glu.
Growth profiles and competition assays indicated that adaptation to a given environment was often associated with improvements in the alternative environments (Table S2, Supporting information, Fig. 4). Phenotypic evolution in a given environment is constrained by selective pressures that need not to apply in alternative environments. Therefore, we expected the correlated phenotypic responses in the alternative environments to be more variable than the direct response in the evolution environment. Surprisingly, this was not the case (Wilcoxon rank-sum test on the coefficient of variation; $\mu_{\text{max}}$: $\chi^2 = 1.9, p = 0.17$; fitness: $\chi^2 = 0.2, p = 0.69$; Table S3, Supporting information).

Next, we tested whether genetic parallelism (i.e., whether clones shared or not mutated genes) and evolutionary history (i.e., whether clones evolved or not in the same evolution environment) affected the correlated responses. Correlated responses to evolution may be expected to be more similar for clones with mutations in identical genes, even if they evolved in different environments, as long as the phenotypic effects of individual mutations affecting a given gene are similar and epistatic interactions between mutations in the different genes do not entirely mask the effect of individual mutations. Similarly, correlated responses to evolution may be expected to be more similar for clones that evolved in identical environments, even if they had mutations in different genes, if the evolved mutational pathways resulted in phenotypic parallelism not only in the evolution environment but also under a wider range of environments. We measured the variability of the correlated responses in each alternative environment for all pairs of clones and used a GLM to distinguish the contribution of genetic parallelism and evolutionary history to the correlated responses to evolution. For pairs of clones that evolved in the same evolution environment we considered the three alternative environments, while for pairs of clones that evolved in two different evolution environments we considered the two shared alternative environments. We tested whether sharing or not mutated genes and evolving or not in identical evolution environments...
influenced the phenotypic variability between clone pairs (Table 2). The interaction between
the two factors was significant for both $\mu_{\text{max}}$ and fitness phenotypes (Table 2), indicating that
the impact of genetic parallelism on phenotypic variability was different whether the clones
evolved in identical or different evolution environments. The variability of the correlated
responses was lower for pairs of clones that evolved in the same environment (Fig. 5).
Moreover, clones with different evolutionary history had more similar correlated responses
when they shared mutated genes (Fig. 5a, b). However, for clones that evolved in identical
evolution environments, the variability of correlated responses was unrelated to the level of
genetic parallelism (Fig. 5c, d).

Discussion

We propagated four replicate populations of *E. coli* B in each of four different environments
for 1000 generations. During this time all populations adapted to their evolution environment.
Adaptation involved a high level of genetic parallelism, and 60% of the identified mutations
affected eight genes that were modified in parallel in at least two populations. Four of these
genes, *argR*, *spoT*, *rho*, and *nadR*, were modified repeatedly across environments, although
no single gene was mutated in all environments. This result suggests distinct, but overlapping,
selective pressures in the different environments. Moreover, the combination of mutated
genes was different in the evolved clones sampled from the various environments. Finally, we
found that evolution in each environment was associated with improved correlated phenotypic
responses in alternative environments and that evolved clones from different evolution
environments revealed a higher level of parallel correlated responses when displaying genetic
parallelism.
Evolution was associated with parallel genetic changes in genes specifically involved in the catabolism of the available carbon source \((glpR \text{ and } glpK)\) in only one of the four environments (Gly). Adaptive evolution may therefore involve improvements of substrate specific catabolic pathways, but this not a general trend. Consistent with this, other evolution experiments have found mutations in genes specifically involved in the catabolic pathways of the available carbon sources in populations propagated in the presence of glycerol (Herring et al. 2006) and L-1,2-propanediol (Lee & Palsson 2010), but not in the presence of lactate (Conrad et al. 2009) or glucose (Barrick et al. 2009). We found that genes involved in global transcription regulation were targets of mutations in all four evolution environments, as observed in most evolution experiments (Cooper et al. 2003; Crozat et al. 2005; Herring et al. 2006; Bantinaki et al. 2007; Conrad et al. 2009; Le Gac & Doebeli 2010; Maharjan et al. 2010; Wang et al. 2010; Yu et al. 2010; Conrad et al. 2011; Tenaillon et al. 2012), reflecting the importance of restructuring regulatory networks during evolution (Philippe et al. 2007; Hindré et al. 2012). Even more strikingly, all genes that were mutated in more than one environment encoded global regulators of gene transcription. Six of the eight genetic loci that were repeatedly changed have also been modified by mutations in other evolution experiments, including \(glpK\), \(glpR\), \(spoT\), \(nadR\), \(mreBC\) and \(rho\) (Cooper et al. 2003; Raghunathan & Palsson 2003; Herring et al. 2006; Woods et al. 2006; Ostrowski et al. 2008; Woods et al. 2011; Tenaillon et al. 2012; Herron and Doebeli 2013).

Genetic parallelism is commonly observed among replicate independent populations that are propagated in similar environments. The nature of the mutations affecting target genes is diverse, from identical nucleotide changes being repeatedly substituted in independent viral populations (Wichman et al. 1999) to different types of mutations distributed at various positions within a given gene in replicate bacterial populations (Woods et al. 2006; Bantinaki...
et al. 2007; Crozat et al. 2010). A recent analysis of 115 replicate populations of *E. coli* B adapted to high temperature revealed few identical mutations among populations, although high genetic parallelism at the gene level was detected (Tenaillon et al. 2012). In our study, genetic parallelism at the gene level was associated with different mutations occurring at different positions within genes. For example, *glpR* was affected by two different non-synonymous mutations, a 125-bp deletion in its coding region, and the insertion of an IS1 element in its promoter region. This profile suggests that the mutations inactivated *glpR*.

However, there were two notable exceptions to this pattern. First, different mutations occurred in specific regions of two of the altered genes. All *argR* mutations were localized in the 5’ gene region encoding the DNA-binding domain of ArgR (Sunnerhagen et al. 1997) and all *glpK* mutations mapped in a ~80-bp region at the 5’ end of the gene. Mutations in this *glpK* region have been shown to increase fitness on glycerol-containing medium by reducing the affinity of GlpK for its allosteric inhibitor fructose-1,6-bisphosphate and inhibiting the formation of the GlpK tetramer (Applebee et al. 2011). Based on these observations, we hypothesize that mutations in *argR* and *glpK* modulated specific activities of the encoded proteins. Second, we detected an even higher level of specificity for the mutations that occurred in *rho* and *spoT*, which were repeatedly modified both within and between environments. Hence, not only were independently substituted mutations localized to nearby codons (322 and 324 in *rho*, 207 and 209 in *spoT*), but identical codons were changed in clones evolved in different environments, including codons 322 and 324 for *rho* in Ace and Gly and codon 393 for *spoT* in Gly and Glu. In the Gly environment, codon 393 of *spoT* was changed in all four clones, and in 3 out of 4 in Glu. Cross contamination between replicate populations can be excluded since four different SNPs affected codon 393. Such an extreme level of parallelism, extending to the level of the substituted mutations, has also been reported in bacterial evolution experiments for *glpK* (Applebee et al. 2011), *rho* (Tenaillon et al. 2012).
...depending on the environment, selection may act either at precise identical codons (in Gly and Glu), leading or not to identical amino-acid changes, or at the level of the entire gene (Glc).

In our evolution experiment, spoT was the most commonly mutated gene, being modified in 3/4 environments and 11/16 clones. It has already been observed as being frequently modified during adaptation to glucose environments. In a commonly-used glucose environment, spoT has been affected by a total of 32 non-synonymous mutations, 28 of which involved a unique amino-acid substitution (Cooper et al. 2003; Cooper 2007; Ostrowski et al. 2008; Woods et al. 2011). In contrast, in two environments in this study, seven out of eight clones had mutations affecting the exact same spoT codon. This suggests that the number of potential beneficial mutations in a given gene may vary from one environment to the other. So far, very little is known about the variability of phenotypic effects associated with different beneficial mutations affecting the same gene. Such studies would undoubtedly increase our comprehension of the relationships between genotypes, phenotypes and fitness.

We have shown that populations evolving in identical evolution environments may display parallel phenotypic evolution in alternative environments, as was previously described (Cooper & Lenski 2000; Barrett et al. 2005; Fong et al. 2005). Moreover, we showed that the correlated phenotypic responses were related to both the evolutionary history of the sampled clones, i.e. whether or not they evolved in identical evolution environments, and the genetic parallelism, i.e. whether or not they shared mutated genes. Our results therefore have two implications. First, individuals that have been subjected to similar historical selective pressures tend to occupy similar ecological niches not only in the environment in which they evolved, but also under a wider range of conditions, even without genetic parallelism. Second, when evolutionary history was different, i.e. for clones that evolved in different evolution...
environments, correlated phenotypic responses were related to the level of genetic parallelism.

It therefore suggests that the mutations of the repeatedly modified genes had similar beneficial effects even in different genomic contexts. This finding indicates that although epistatic interactions between mutations are known to considerably alter the effect of individual mutations, they evidently did not entirely mask the individual effects of the mutations under our environmental conditions.

In summary, notwithstanding the relatively small number of genes mutated in more than one environment, our results provide a way to experimentally disentangle the relative contribution of evolutionary history and genetic parallelism on the phenotypic performance in naïve environmental conditions. Taken together, our results indicate that populations sharing historical selective pressures could react similarly to the modification of environmental conditions, even if they do not share mutations in specific genes. To a lesser extent, they also indicate that populations with different evolutionary histories may perform more similarly when facing a new environment if they share mutated genes.

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with heat. Molecular Biology and Evolution, 17, 942-950.


**Data Accessibility:**

Genome sequencing data: ENA Sequence Read Archive (ERP001471)

Fitness, growth, and GLM data: DRYAD data identifier:doi:10.5061/dryad.n2582

**Supporting information**

Additional supporting information may be found in the online version of this article.
Table S1 Phenotypic traits (μ_max and fitness) of the 16 populations during evolutionary time in their respective evolution environments.

Table S2 Phenotypic traits (μ_max and fitness) of the evolved clones sampled from each of the 16 populations in the four environments.

Table S3 Among-clone coefficient of variation in the four environments for the two phenotypic traits μ_max and fitness.

Figure Legends

Fig. 1 Phenotypic trajectories of 16 evolving populations during 1000 generations in their respective evolution environments. Average μ_max (a) and fitness (b) (+/- SEM) are indicated at, respectively, 200-generation intervals, and 300, 600 and 1000 generations for the replicate populations that evolved in the environments Ace (○), Gly (△), Glc (□), and Glu (◇).

Fig. 2 Phenotypic traits of the evolved clones sampled from the replicate populations that were propagated in the environments Ace (○), Gly (△), Glc (□), and Glu (◇). The fitness and growth rate (μ_max) values were measured for one evolved clone, sampled from each population after 1000 generations, in its respective evolution environment. Values were log-transformed and given in the two-dimensional phenotypic space. Each experiment was performed with five-fold replication and values are given +/-SEM. The phenotypic values for the ancestor are (0;0).

Fig. 3 Venn diagrams showing the mutated genes identified in the 16 evolved clones. Each of the 16 ellipses represents one evolved clone sampled from each of the 16 populations. Red, blue, green, and purple ellipses indicate the environments Ace, Gly, Glc, and Glu,
respectively. Overlaps indicate mutated genes shared between clones. Names of genes are written in different colours whether they are affected by mutations in at least two different environments (white), or in only one environment either in a single (black) or in different clones in that environment (colour corresponding to the specific environment).

Fig. 4 Phenotypic traits of the evolved clones sampled from each replicate population both in their respective evolution environment (○) and in the three alternative ones (●). The $\mu_{\text{max}}$ (a to d) and fitness (e to h) values (+/- SEM) of each evolved clone are given in each of the Ace, Gly, Glc, and Glu environments (indicated from left to right on the x-axes). The evolution environment from which each set of four clones was sampled is indicated at the top of each panel: Ace (a, e), Gly (b, f), Glc (c, g), and Glu (d, h). Note that y-axis scales vary.

Fig. 5 Among-clone phenotypic variability in the alternative environments. Each small hollow symbol (○) corresponds to a comparison between a pair of clones in one of the alternative environments. The y-axis shows the phenotypic variability (mean squares) ranked within each alternative environment, a high rank value indicating that the pair of clones displays very different phenotypes (due to tied ranks, not all of them are seen on the figure). Phenotypic variability is given for $\mu_{\text{max}}$ (a, c) and fitness (b, d) values for pairs of clones that evolved in different (a, b), and identical (c, d) environments. Median ranks (●) as well as first and third quartiles (---) are indicated.

Tables

Table 1 Mutations identified in the 16 evolved clones sampled after 1000 generations of evolution in four different environments.
The name of the genes carrying mutations are given, together with their ECB numbers (Jeong et al. 2009). For intergenic mutations, the name of the two surrounding genes is given.

Table 2 Variability of the correlated phenotypic responses for each pair of clones in the alternative environments as a function of genetic parallelism (shared mutated genes or not) and evolutionary history (same evolution environment or not). Likelihood ratio $\chi^2$ tests on ordinal logistic regressions (GLM, Material and methods) were performed with the among-clone variability (Mean square among clones) of the $\mu_{\text{max}}$ and fitness values (ranked within each alternative environment) taken as ordinal response variables. Evolution in identical environments and level of genetic parallelism among clones were taken as explanatory variables.

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<th>Source</th>
<th>DF</th>
<th>$\chi^2$</th>
<th>P-value</th>
</tr>
</thead>
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<td>73.07</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Evolutionary history</td>
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<td>33.88</td>
<td>&lt;0.0001</td>
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<tr>
<td>Model</td>
<td>df</td>
<td>Sum of Squares</td>
<td>P-value</td>
</tr>
<tr>
<td>------------------------------------------------------</td>
<td>----</td>
<td>----------------</td>
<td>---------</td>
</tr>
<tr>
<td>Genetic parallelism</td>
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<td>3.55</td>
<td>0.0596</td>
</tr>
<tr>
<td>Environment X Parallelism</td>
<td>1</td>
<td>14.61</td>
<td>0.0001</td>
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<tr>
<td>Fitness</td>
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</tr>
<tr>
<td>Whole model</td>
<td>3</td>
<td>24.56</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Evolutionary history</td>
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<td>14.60</td>
<td>0.0001</td>
</tr>
<tr>
<td>Genetic parallelism</td>
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<td>0.08</td>
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<tr>
<td>Environment X Parallelism</td>
<td>1</td>
<td>4.60</td>
<td>0.0320</td>
</tr>
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</table>

Indicates whether the model based on the two explanatory variables as well as their interaction significantly explained the among clone variability.
(a) 

Log ($\mu_{\text{max}}$) vs. Generations

(b) 

Log (Fitness) vs. Generations
(c) Fitness variability (ranked)

(d) Fitness variability (ranked)
Supporting information

Evolutionary history and genetic parallelism affect correlated responses to evolution

Mickael Le Gac, Tim Cooper, Stéphane Cruveiller, Claudine Médigue, and Dominique Schneider

This file includes:

**Table S1** Phenotypic traits ($\mu_{\text{max}}$ and fitness) of the 16 populations during evolutionary time in their respective evolution environments.

**Table S2** Phenotypic traits ($\mu_{\text{max}}$ and fitness) of the evolved clones sampled from each of the 16 populations in the four environments.

**Table S3** Among-clone coefficient of variation in the four environments for the two phenotypic traits $\mu_{\text{max}}$ and fitness.
**Table S1** Phenotypic traits ($\mu_{\text{max}}$ and fitness) of the 16 populations during evolutionary time in their respective evolution environments

<table>
<thead>
<tr>
<th>Population</th>
<th>0</th>
<th>200</th>
<th>400</th>
<th>600</th>
<th>800</th>
<th>1000</th>
<th>0</th>
<th>300</th>
<th>600</th>
<th>1000</th>
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<td>Ace_1</td>
<td>16.8±0.10</td>
<td>2.08±0.32</td>
<td>1.99±0.09</td>
<td>2.05±0.22</td>
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<td>Ace_2</td>
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<td>2.42±0.13</td>
<td>2.08±0.21</td>
<td>2.36±0.10</td>
<td>2.24±0.18</td>
<td>2.36±0.06</td>
<td>1.94±0.05</td>
<td>1.87±0.19</td>
<td>2.40±0.22</td>
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</tr>
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<td>Ace_3</td>
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<td>2.17±0.02</td>
<td>2.37±0.21</td>
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<td>2.28±0.14</td>
<td>1.73±0.14</td>
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<tr>
<td>Ace_4</td>
<td>1.58±0.08</td>
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<td>1.71±0.05</td>
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<td>1.91±0.03</td>
<td>1.79±0.15</td>
<td>1.98±0.09</td>
<td>1.51±0.05</td>
<td>1.84±0.05</td>
<td>1.68±0.10</td>
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<tr>
<td>Gly_4</td>
<td>1.17±0.01</td>
<td>1.66±0.01</td>
<td>1.95±0.20</td>
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<td>2.08±0.08</td>
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<td>1.61±0.09</td>
<td>1.94±0.22</td>
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<tr>
<td>Glc_1</td>
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<td>1.73±0.08</td>
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<td>Glu_3</td>
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* $\mu_{\text{max}}$ and fitness values are presented as log-transformed in Figure 1 but not in Table S1.
Table S2: Phenotypic traits (μ<sub>max</sub> and fitness) of the evolved clones sampled from each of the 16 populations in the four environments

<table>
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<th>Environment</th>
<th>Population</th>
<th>μ&lt;sub&gt;max&lt;/sub&gt;</th>
<th>fitness</th>
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<tr>
<td>Ace</td>
<td>Ace_1</td>
<td>2.71+-/-0.07</td>
<td>1.36+-/-0.06</td>
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<td></td>
<td>Ace_2</td>
<td>2.78+-/-0.07</td>
<td>1.27+-/-0.04</td>
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<tr>
<td></td>
<td>Ace_3</td>
<td>2.46+-/-0.17</td>
<td>1.31+-/-0.06</td>
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<tr>
<td></td>
<td>Ace_4</td>
<td>2.91+-/-0.06</td>
<td>1.34+-/-0.08</td>
</tr>
<tr>
<td>Gly</td>
<td>Gly_1</td>
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<td>Very slow growth</td>
</tr>
<tr>
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<td>Gly</td>
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<td>Very slow growth</td>
<td>Very slow growth</td>
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<tr>
<td>Gly</td>
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<td>1.70+-/-0.09</td>
<td>1.17+-/-0.07</td>
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<td>Glc</td>
<td>Glc_3</td>
<td>1.68+-/-0.11</td>
<td>1.22+-/-0.03</td>
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<td>Glu_3</td>
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<td>Ace</td>
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<tr>
<td>Gly</td>
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<td>1.79+-/-0.10</td>
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<td>1.63+-/-0.04</td>
</tr>
<tr>
<td>Glu</td>
<td>Glu_4</td>
<td>1.57+-/-0.09</td>
<td>1.32+-/-0.02</td>
</tr>
<tr>
<td>Ace</td>
<td>Ace_1</td>
<td>1.16+-/-0.11</td>
<td>0.95+-/-0.17</td>
</tr>
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<td>Ace_2</td>
<td>1.25+-/-0.03</td>
<td>1.10+-/-0.09</td>
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<td>Ace_3</td>
<td>1.26+-/-0.10</td>
<td>1.09+-/-0.05</td>
</tr>
<tr>
<td>Ace</td>
<td>Ace_4</td>
<td>1.17+-/-0.04</td>
<td>0.97+-/-0.19</td>
</tr>
<tr>
<td>Gly</td>
<td>Gly_1</td>
<td>1.41+-/-0.07</td>
<td>2.14+-/-0.50</td>
</tr>
<tr>
<td>Gly</td>
<td>Gly_2</td>
<td>1.53+-/-0.08</td>
<td>1.55+-/-0.17</td>
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</table>
The $\mu_{\text{max}}$ and fitness values are given (+/-SEM) for each evolved clone sampled from each of the 16 evolving populations in each of the four different environments.

* $\mu_{\text{max}}$ values are presented as log-transformed in Figure 2 but not in Table S2.

** As no quantitative values could be obtained for these measures, they were not included in the statistical analyses.
Table S3 Among-clone coefficient of variation in the four environments for the two phenotypic traits μ\text{max} and fitness

<table>
<thead>
<tr>
<th>Evolution environment</th>
<th>Alternative environment</th>
<th>Coefficient of variation</th>
<th>μ\text{max}</th>
<th>fitness</th>
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<td>0.15</td>
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<td>Gly</td>
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<td>0.04</td>
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<tr>
<td></td>
<td>Glc</td>
<td></td>
<td>0.03</td>
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<td>0.04</td>
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<tr>
<td>Gly</td>
<td>Ace</td>
<td>Very slow growth</td>
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<td>0.02</td>
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<td>0.05</td>
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<td>Glu</td>
<td></td>
<td>0.06</td>
<td>0.05</td>
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<tr>
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<td>Ace</td>
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<td></td>
<td>Glu</td>
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<td>0.13</td>
<td>0.03</td>
</tr>
</tbody>
</table>

As no quantitative values could be obtained for these measures, they were not included in the statistical analyses.