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Differential stress resistance and metabolic traits underlie coexistence in a sympatrically evolved bacterial population

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Summary

Following intermittent batch growth in Luria–Bertani (LB) broth for about 1000 generations, differentially evolved forms were found in a population of *Escherichia coli* cells. Studies on this population revealed the emergence of key polymorphisms, as evidenced by analysis of both whole genome sequences and transcription analysis. Here, we investigated the phenotypic nature of several key forms and found a remarkable (interactive) coexistence of forms which highlights the presence of different ecological roles pointing at a dichotomy in: (i) tolerance to environmental stresses and (ii) the capacity to utilize particular carbon sources such as galactose. Both forms differed from their common ancestor by different criteria. This apparent coexistence of two diverged forms points at the occurrence of niche partitioning as a consequence of dichotomous adaptive evolution. Remarkably, the two forms were shown to continue to coexist – in varying ratio's – in an experiment that cycled them through periods of nutrient feast (plentiful growth substrates) and famine (growth-restrictive – stress conditions). The results further indicated that the equilibrium of the coexistence was destroyed when one of the parameters was high tuned, jeopardizing the stability of the coexisting pair.

Introduction

Many (natural and artificial) environments expose bacteria to fluctuating conditions, either in space or in time. Bacteria modulate their genomic and phenotypic characteristics to cope with such changes in their surroundings. Ecological theory posits that monomorphic populations in nature come about as a result of intense competition for limiting resources (Gause, 1934; Tilman, 1990). In contrast and as a result of niche partitioning, coexistence of phenotypically distinct bacterial populations in sympatric conditions may also occur (Rosenzweig *et al*., 1994; Kinnersley *et al*., 2009; Herron and Doebeli, 2013). For instance, initially, isogenic populations of *Escherichia coli* in glucose-limited continuous culture diversified into distinct metabolic types (Helling *et al*., 1987; Maharjan *et al*., 2006). Such an effect was recently also found for *E. coli* developing in complex growth medium (Puentes-Téllez and van Elsas, 2014). In another experiment, replicate *E. coli* populations diversified into two coexisting metabolic types due to a diauxic shift in serial-batch culture fed with a mixture of two substrates, glucose and acetate (Friesen *et al*., 2004; Herron and Doebeli, 2013).

The occurrence of unexplored (vacant) niches in the aforementioned habitats may thus constitute a key driver of the rapid emergence of novel bacterial types in initially homogeneous populations (Rainey and Travisano, 1998; MacLean *et al*., 2005; Puentes-Téllez *et al*., 2013), in which frequency-dependent selection may be involved (Friesen *et al*., 2004; Tyerman *et al*., 2005). One may consider complex environments with nutrient mixtures to offer spatial 'structure' with fine-scale variation (Kassen, 2002). Evolution in a complex environment enhances the likelihood of diversification (Buckling *et al*., 2003; Spencer *et al*., 2008; Herron and Doebeli, 2013). Although diversification may initially be transient (Rainey and Travisano, 1998), the effect of interactions between the evolved types may increase the stability of heterogeneous communities. Thus, in complex environments, there are increased chances of establishment followed by maintenance of heterogeneity as a result of interactions that spur the coexistence of successful emerged types (Helling *et al*., 1987; Rosenzweig *et al*., 1994).

In our laboratory, a population of *E. coli* cells growing in LB broth in sequential-batch cultures under constant oxygen (during ~ 1000 generations) was recently shown

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to diversify into multifold evolved forms. Two distinct metabolic types were identified after analysis of multiple colonies from the evolved population. Initial studies of these revealed that colony morphology correlated with some distinct genotypes and phenotypes (Puentes-Téllez *et al*., 2013; 2014). Here, we further explore the ecological nature of selected divergent types that apparently maintained a coexisting interaction. Daily transfers from old cultures to fresh media followed by outgrowth exposed the populations to alternating periods of nutrient 'feast and famine'. Thus, we assessed the basis of the potentially coexisting phenotypes and interrogated the system as to the stability of coexistence in the face of habitat fluctuations (Experimental setup; Supporting Information Fig. S1). We obtained evidence for the contention that adaptive evolution in the sympatric *E. coli* population resulted in the emergence of two major forms showing phenotypes that enabled two clearly different ecological strategies. The two forms coexisted at different ratios under the fluctuating nutrient feast/famine conditions that were applied.

Results and discussion

Divergence in survival capabilities under osmotic, heat and acidity stresses

To assess whether the survival capabilities had diverged between different forms in the evolved *E. coli* population, we exposed three selected forms ('*a*', '*b*' and '*c*', representing different colony morphologies), next to the ancestor, to osmotic, high-temperature and acidity stresses using cells from two different growth stages (exponential growth and stationary phase). Figure 1 shows the changes in viability of the cells (as percentage survival) during the first 4 h of each of the challenges.

We observed remarkable differences between the survival levels of cells from both growth stages. First, the responses of exponential-phase cells to osmotic and high-temperature stress conditions were quite diverse (Fig. 1A). After 4 h under both conditions, there was a significant dispersion among the values exhibited by all strains [one-way analysis of variance (ANOVA), $P < 0.001$]. Remarkably, the ancestor was the better survivor, maintaining the population rather stable during the first 2 h of challenge. This stress resistance persisted until the end of the heat challenge. However, in the osmotic stress treatment, after 3 h, the ancestral populations revealed a decreasing trend. A second striking observation was that exponential-phase cells of forms '*b*' and '*c*' survived both osmotic and heat stresses to similar extents ($P > 0.05$). Moreover, these forms were more sensitive to osmotic than to heat stress, revealing greater decreases in viable cell numbers. Overall, these form '*b*' and '*c*' responses were significantly different from those of the ancestor and of form '*a*' ($P < 0.05$). The form that the populations showed decreases to around 2% of the initial population size after the first 2 h under osmotic stress. However, from this point on, the survival levels became progressively higher, with about 10% of the initial cell populations surviving at the end of the challenge. Apparently, part of the form '*a*' population recovered its ability to grow after a short period of osmotic stress, resulting in a final population size similar to the one of the ancestral strain ($P > 0.05$). In contrast, as from the first hour of heat challenge, form '*a*' showed a dramatic and progressive decay of culturable forms, without any recovery. For instance, after 2 h, only 1% of the population was still viable. This was significantly different from the dynamics of the ancestral strain ($P < 0.05$).

The stationary-phase cells of all forms (including the ancestor) revealed similar survival when confronted with the osmotic and heat stress challenges (one-way ANOVA; 4 h; $P > 0.05$) (Fig. 1B). However, whereas osmotic stress reduced the population sizes to less than 10% of the initial one after the first hour, heat stress only did so to a limited extent (about 50% of loss was observed up to the third hour). The similar response patterns found across all tested forms under osmotic and heat stress may relate to a similar activation of key proteins, potentially the ones that also act upon starvation for energy/carbon sources. Upon entering stationary phase, bacterial cells are known to start up the production of a large set of 'starvation proteins', which change the physiological status of the cells. This results in physiologically strongly altered cells and cross-protection against challenges with stressors like osmotic tension, heat, oxidative stress and the pressure of antibiotics (Groat *et al*., 1986). A major modulator of this stress-tolerant state in bacteria is the product of the *rpoS* gene, which allows the cells to survive actual stresses as well as those that are not yet present (Hengge-Aronis, 2002; Dragosits *et al*., 2013).

With respect to acidity (pH 3.0) stress, all exponential-phase forms revealed similar decay responses, being sensitive as from the first hour of challenge (one-way ANOVA; 4 h; $P > 0.05$) (Fig. 1A). However, with respect to the stationary-phase cells, the survival values were different (one-way ANOVA; 4 h; $P < 0.001$). Moderate acid tolerance was found in stationary-phase cells of form '*a*' (and ancestor; Fig. 1B), whereas the population sizes of similar form '*b*' and '*c*' cells decreased dramatically already after 1 h of challenge. Thus, given their enhanced capacity to resist acidity, form '*a*' cells may occupy a specific niche in the evolved population. Our previous work also showed that forms '*b*' and '*c*' had similar metabolic behaviour, including the propensity to efficiently breakdown several carbohydrates under aerobic conditions. This type of active metabolism can generate acetate, which can be

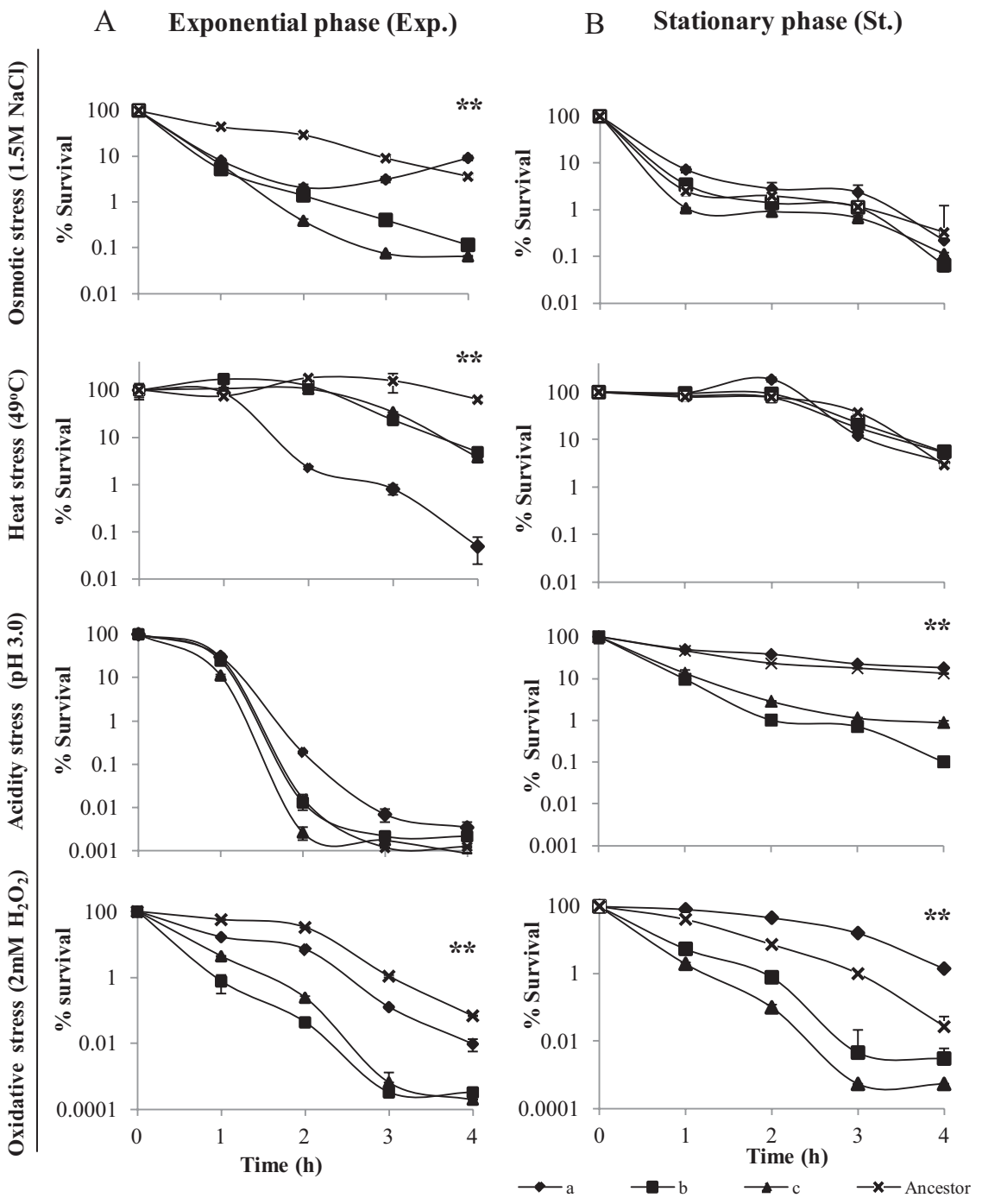

Fig. 1. Survival test. Forms '*a*', '*b*' and '*c* ' and the ancestor were challenged to four different stress conditions added to LB. Osmotic stress (1.5 M NaCl), Heat stress (49°C), oxidative stress (2 mM H_2O_2) and pH stress (pH 3.0). Challenge took place during 4 h and after pre-adaptation [5 h and 18 h – (A) Exponential-phase cells and (B) stationary-phase cells respectively]. Logarithmic plots show the percentage of loss relative to the initial (100%) population. All results are reported as the mean + SD of three replicates. Significant differences are reported at 4 h of challenge, one-way ANOVA: $^{**}P < 0.001$. Osmotic challenge: Exp.: $F(3,8) = 86,25$; St: $F(3,4) = 3.02$. Heat challenge: Exp: $F(3,8) = 20,09$; St: $F(3,8) = 2.94$. Acidity challenge: Exp: $F(3,4) = 3.64$; St:$F(3,4) = 87.59$. Oxidative challenge: Exp: $F(3,8) = 47.33$; St: $F(3,8) = 24.70$.

reincorporated into central metabolism by particular cells in the population (Wolfe, 2005). Thus, the potentially fast and active metabolism occurring in forms '*b*' and '*c*' may have incited an accumulation of acetate in the medium and a consequent reduction of the pH (Wolfe, 2005). Form '*a*' has been found to be able to consume and grow on acetate (Puentes-Téllez and van Elsas 2014), and hence there may have been evolutionary pressure favouring form '*a*', as it maintained the ancestral capability to cope with acid conditions and could use any released acetate. The former ability was apparently lost by forms '*b*' and '*c*' and so form '*a*' might serve an acetate/acidity vacuum-cleaner role allowing both '*b*' and '*c*' to survive.

Oxidative stress resistance in forms 'a', 'b' and 'c'

After 4 h of oxidative stress, all forms in both physiological states revealed significant differences between them (one-way ANOVA; $P < 0.001$). We first examine the differential responses of populations of form '*a*' on the one hand versus forms '*b*' and '*c*' on the other hand (Fig. 1). Populations of the latter forms constituted the more sensitive forms at both growth phases, differing significantly from the more resistant ones of form '*a*' and the ancestor ($P < 0.05$). Then, the response of form '*a*' differed significantly from that of the ancestor, as in both exponential- and stationary-phase cells, and results differed significantly ($P < 0.05$).

Regarding the stationary-phase cells, whereas the ancestor's populations declined rapidly after the first hour of challenge (declining to about 40% of the population), form '*a*' revealed a reduction to just about 80%. After 3 h, the ancestor was practically eliminated, whereas form '*a*' still revealed a population of about 20% of the initial number. This striking uplift in the tolerance of form '*a*' to oxidative stress indicated a particular adaptive path and the putative occurrence of oxidative stress as a selector of this phenotype defining the niche.

Bacteria under stress may convert to a transient mutator state, in which they undergo enhanced genetic change, which may spur adaptive evolution (Foster, 2007). In our previous study (Puentes-Téllez *et al.*, 2013), a suite of 'inconsistent' (not occurring in all forms) mutations was found in the sympatric *E. coli* population. Concerning the three forms in the population, form '*a*' was found to carry a raised number of mutations (67), whereas forms '*b*' and '*c*' had 47 and 45 changes on the genome respectively. Supporting Information Table S2 lists the inconsistent mutations present in the three forms. Interestingly, when the mutations between forms '*b*' and '*c*' are compared, we observed only few differences between them, i.e. form '*b*' has a unique mutation in the *yhhZ* gene (encoding a protein of unknown function, Rudd, 2000), whereas form '*c*' had two mutations in different loci of the *rfc* gene (encoding a protein involved in the production of lipopolysaccharides in the outer membrane, Lukomski *et al.*, 1996) and a mutation in *yfiF* (encoding a protein of unknown function, Rudd, 2000). We do not know whether these differences matter, but this should be further evaluated. However, when looking for high-impact changes (at the level of regulation), it was found that among the genetic commonalities between forms '*b*' and '*c*', one mutation (not present in form '*a*') was present in the major stress regulator *rpoA* (encoding the α subunit of RNA polymerase, RpoA). The mutation was located in codon 294 and leads to a change of the codon from asparagine to histidine in RpoA. It concerned the OxyR protein contact site in the C-terminal region of RpoA. Interestingly, OxyR is a positive regulator of hydrogen peroxide (oxidative stress)-inducible genes in *E. coli*, and such activation has not been found in mutants containing a C-terminal-truncated α subunit (Tao *et al.*, 1993; 1995). Thus, a changed efficiency of OxyR protein synthesis might be involved in the observed reduced resistance to oxidative stress in both forms '*b*' and '*c*'. Functional annotation of the 'unique' mutated genes in form '*a*' using DAVID v. 6.7 (Huang *et al.*, 2009) resulted in the finding of two main clusters of GO (gene ontology) terms: i.e. (i) genes-encoding proteins involved in membrane processes and (ii) genes-encoding proteins involved in DNA-binding processes. These processes are likely involved in the oxidative stress resistance observed in this form. On top of that, the lack of the *rpoA* mutation in form '*a*' also supported the physiology of this form towards an oxidative stress resistance advantage.

Overall, we observed fairly similar declines of all forms under the four tested stress conditions (osmotic, heat, acidity and oxidative stresses), with major differences being seen between two groups of forms; i.e. (i) form '*a*' and the ancestor versus (ii) forms '*b*' and '*c*'. Apparently, the evolved genomic/regulatory systems of forms '*b*' and '*c*' did not include important strategies to withstand stress, whereas the ancestral capacity to deal with stress was kept, and potentially improved, by form '*a*'.

Dynamics of coexistence between metabolic types a and c

Two metabolic types among the selected forms '*a*', '*b*' and '*c*' have previously been selected, i.e. 'metabolic types' *a* and *c*, with *c* representing both forms '*b*' and '*c*' (Puentes-Téllez and van Elsas, 2014). Whereas type *c* showed a fast carbohydrate consumption ability, type *a* (a slow grower on major substrates of LB), had developed the ability to consume acetate. Assessment of the growth of both types separately in LB broth showed faster exponential growth ($\mu = 0.26$) of type *c* and, consequently, an earlier entrance into stationary phase, compared with type

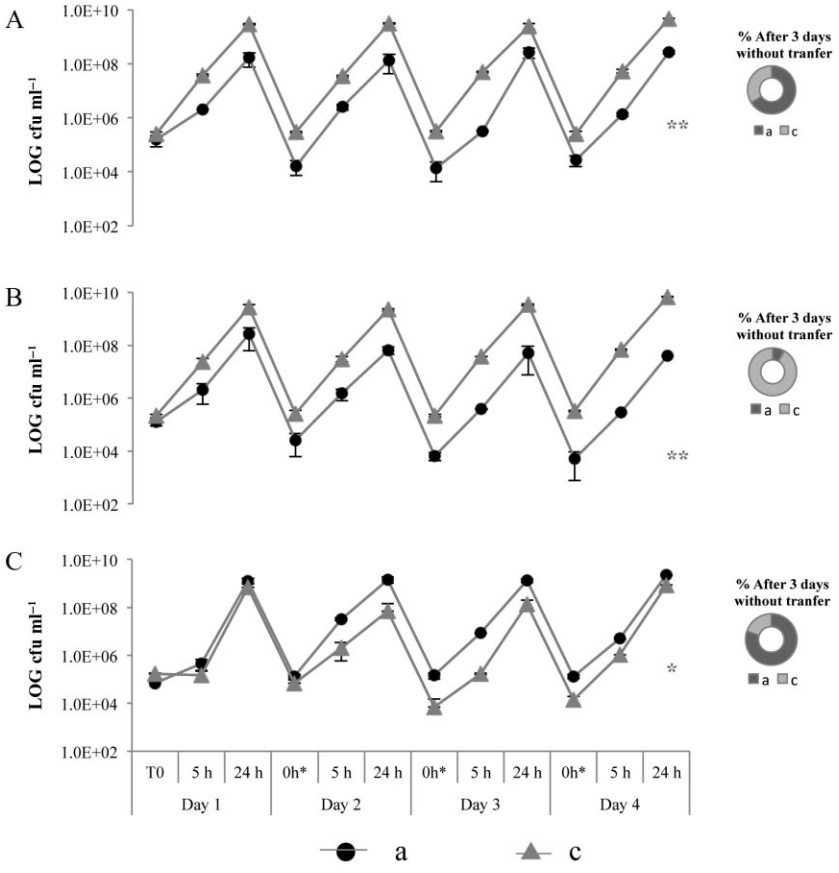

Fig. 2. Coexistence experiment. Types *a* and *c* growing together in (A) LB broth, LB broth supplemented with (B) galactose (0.3%) and (C) H_2O_2 (0.3 mM). *Predicted cfu per ml at 0 h, calculated from dilution (1:10 000). Error bars show standard deviation. In boxes: percentage of each type following day 4 and after 3 days without any transfer. Significant difference calculated at after day 4, one-way ANOVA $^{**}P < 0.001$, $^{*}P < 0.05$. LB: $F(1,6) = 32.90$; LB + galactose: $F(1,6) = 1108.7$; LB + H_2O_2: $F(1,6) = 207.2$.

a ($\mu = 0.22$; $P < 0.05$). Type *a* thus revealed a longer lag phase, resulting in a later exponential phase and also a later stationary phase (Puentes-Téllez and van Elsas, 2014).

On the basis of the current data, we surmised that tolerance to stress constitutes another major factor that differentiates types *a* and *c*. We thus decided to test the hypothesis that these two forms are co-selected in a serial-batch culture cycling regimen, in which conditions stimulating rapid growth in LB are interspersed with those offering starvation (and possibly other) stress to the cell population. Growth was assessed over four daily transfers using mutants of each type having different markers and starting with equal cell densities per type. Figure 2A shows the dynamics of the mixed populations, as colony-forming unit (cfu) ml^{-1} [including samples taken at two growth phases: exponential (5 h) and stationary phase (24 h)]. After 5 h of growth in each new condition, type *c* revealed a consistent advantage over type *a* (all *t*-tests; 5 h after each transfer; $P < 0.05$). However, although lower in population size, type *a* was invariably maintained in the population and veered back in relative abundance after the stationary phase. This dynamic 'group behaviour', with a significant difference between the two types, remained until the last day of coexistence (one-way ANOVA, $P < 0.001$) with a fairly steady behaviour during the competition and a final type:type ratio of 20:1. Thus, there was no consistent ecological advantage or disadvantage for either of the two types in LB broth. Rather, a balanced equilibrium appeared to exist between them, which supports the idea of coexistence of two ecologically divergent types through the feast-famine cycling regimen in LB.

Then, we aimed to observe the dynamics of the population in relation to the specific properties and stability of the coexisting pair. The stability of coexistence was tested by growing both metabolic types in galactose- or H_2O_2-supplemented LB broth ['LB + galactose' (0.3%) or 'LB + H_2O_2' (0.3 mM)] as 'inducer' environments. The results show clear differences in effects of these two environments on the two coexisting types. Type *c* was a clear winner, in terms of population size, in LB + galactose, ending up at d4 with a very significantly raised population size when compared with type *a* (one-way ANOVA; $P < 0.001$) (Fig. 2B), establishing a ratio of 170:1. In contrast, type *a* virtually took over the population in LB + H_2O_2 (one-way ANOVA; d4; $P < 0.05$), establishing a ratio of 3:1 (Fig. 2C). However, in spite of the great

influence of the introduced environmental factor, LB supported the maintenance of a background of each type. When growing separately in LB + galactose, a significant advantage of type *c* [$\mu = 0.310 \pm$ standard deviation (SD) 0.009] over type *a* ($\mu = 0.234 \pm$ SD 0.005) ($P < 0.001$) was also found, whereas in LB + H_2O_2, there was an advantage of type *a* ($\mu = 0.245 \pm$ SD 0.005) over type *c* ($\mu = 0.185 \pm$ SD 0.014) ($P < 0.05$). Supporting Information Table S3 shows the individual growth rates ($\pm$ SD) obtained during growth in LB supplemented with galactose and H_2O_2. These results confirm the advantage of type *c* in dealing with carbon sources and the advantage of type *a* to deal with stress.

We then sought to observe if a longer stationary phase (and a posterior death phase) would have a selective effect on the types present in the population. In LB and LB + H_2O_2, type *a* almost took over the population (66% and 80.2% respectively) after 3 days of culturing without transfer. Exhausted medium presumably allows the form with raised stress resistance to dominate the population. Interestingly, in LB + galactose, type *a* did not persist well, as 91.9% of the population consisted of type *c* cells. A detrimental end-effect of the galactose added to LB on type *a* was apparent from the beginning of the experiment. As type *c* has a higher affinity for the uptake of carbohydrates, the high concentration of galactose present in the medium may have been selective for type *c* and consequently detrimental to *a*. In addition, after 5 h of growth at d1 (initial pH in all transfers adjusted to 6.9) the pH in the LB broth had dropped to 6.7 ± 0.03 and in LB + H_2O_2 to 6.72 ± 0.01. In LB + galactose, the pH had dropped even further, to 6.20 ± 0.02. Apparently, the accumulation of intolerable levels of acidity (possibly acetic acid) in the galactose-containing medium did not allow type *a* to use this carbohydrate efficiently. Thus, galactose present at each subsequent transfer may have maintained a negative effect on type *a*. In other words, surpassing the tolerance level of a sensitive type in the habitat may break down equilibrium and coexistence, resulting in the form with the highest intrinsic growth rate to displace the other one.

Relative fitness during coexistence in 'inducer environments'

Relative fitness (*w*) of the forms was calculated at the end of the coexistence experiment (d4) using the ratio of their Malthusian parameters. Supporting Information Figure S2 shows the fitness of type *a* relative to type *c*. The null hypothesis is that the mean fitness of the evolved forms is 1.0. The metabolic advantage of type *c* in both LB and LB + galactose was confirmed ($w = 0.78 \pm 0.003$ and $w = 0.51 \pm 0.062$), as well as that of type *a* under oxidative stress (H_2O_2) conditions ($w = 1.20 \pm 0.027$). The two 'inducer environments' are clearly differentially selective. Thus, our results suggest differential niche occupation and the presence of distinct ecological roles within the population.

Differential transcript levels of central metabolism during exponential growth phase

We sought to observe the putative differences in transcription patterns between the 'metabolic types' *a* and *c*. Thus, the activities of specific genes and pathways were tested during growth in LB broth and in the two 'inducer environments'. For this, eight primer sets were used to target cDNA generated from purified messenger RNA (Mrna) obtained from exponential- and stationary-phase cells. We found that the data based on copy numbers per ng of mRNA were comparable with those analyzed as copy number of mRNA/cell. Thus, genes related to carbohydrate metabolism and aerobic respiration (*galE*, *treB*, *ptsH*, *cyoA*, *acs*) were tested (Fig. 3A). There were striking differences between the expression levels in the three environments. As part of the gal operon, *galE* catalyzes the inter-conversion of uridine diphosphate (UDP)-galactose and UDP-glucose during galactose catabolism. A consistent point mutation was found previously in the evolved forms '*a*', '*b*' and '*c*' of this population, reverting a mutated *galE* gene in the ancestral strain (Puentes-Téllez *et al.*, 2013). Moreover, a point mutation in the galactose repressor *galR* was observed. These evolutionary changes were likely allowing galactose metabolism (Puentes-Téllez *et al.*, 2013). The metabolic advantage of type *c* over type *a* when using galactose was confirmed by the increased mRNA copies in both LB and galactose-supplemented medium ($P < 0.05$ and $P < 0.01$) during the exponential growth phase. Moreover, in this phase, there was higher mRNA copy number of gene *treB* in the population of type *c* growing in LB ($P < 0.01$). The product of gene *treB* is involved in the transport of trehalose, moving trehalose 6-phosphate into the cytoplasm (Horlacher and Boss, 1997). It belongs to the phosphotransferases system (PTS) group, a group of enzymes involved in sugar transportation (Klein *et al.*, 1995). Interestingly, there is no difference in the inducer (galactose) environment, suggesting an equal preference from type *c* relative to type *a* for trehalose.

Another PTS, the *ptsH* gene product, is involved in non-sugar-specific uptake and transport in bacteria, resulting in phosphorylation of a number of carbohydrates in *E. coli* (Anderson *et al.*, 1971; Kornberg, 1986; Postma and Lengeler, 1993). Our results revealed a significant difference in the number of *ptsH* transcripts between the two types during exponential phase in the three tested environments. Type *c* shows a higher number of transcripts compared with type *a* ($P < 0.05$). On the other

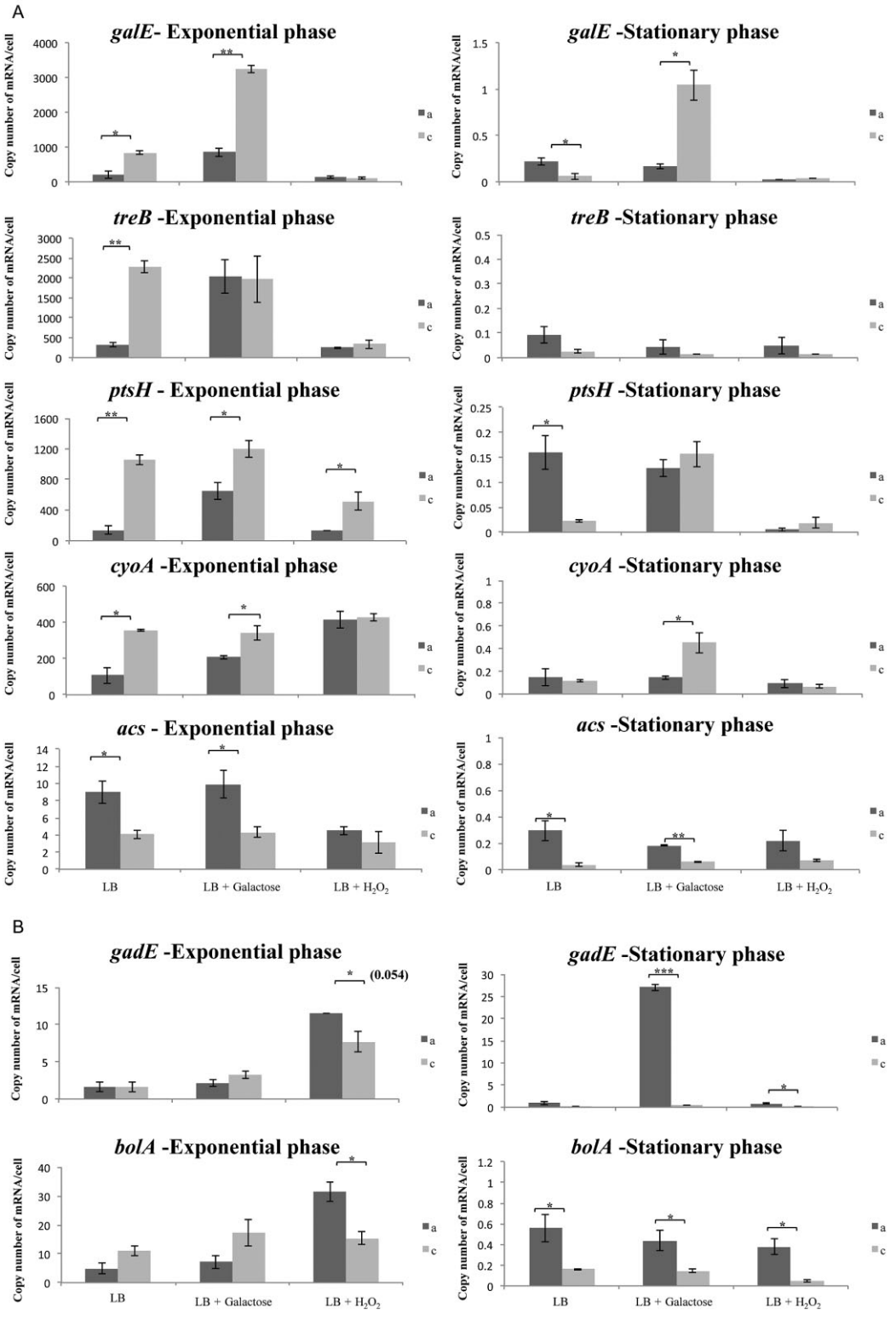

Fig. 3. Distinct transcriptional levels of central metabolism and stress-related genes.
A. Copy number of mRNA per cell of five selected central metabolism-related genes. mRNA from cells in two different growth stages and grown under three different environments (LB broth and LB supplemented with 0.3% of galactose or with 0.3 mM of H_2O_2) was used in the real-time PCR reaction ($n = 4$).
B. Results obtained in two stress response–related genes (*gadE* and *bolA*) ($n = 4$). Two-tailed *t*-test was used for comparisons. Three different cut-off *P* values were established in order to observe with more detail differences between the types ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

hand, being an indicator of higher respiration and faster metabolism (Nakamura *et al*., 1990), the raised levels of *cyoA* transcripts (compared with type *a*) confirmed the raised activity of type *c* during exponential phase ($P < 0.05$). The early activation of carbohydrate-related genes confirmed the fine-tuning of type *c* with respect to rapid carbon metabolism and respiration. On another note, the transcript levels of the *acs* gene in cells from exponential and even stationary phase were significantly raised in type *a* as compared with type *c* in the environments were type *c* has a growth advantage (LB and LB + galactose). The *acs* gene produces acetyl CoA synthetase (ACS), bringing acetate into the central metabolism (Brown *et al*., 1977). Thus, the consumption of acetate was confirmed by a higher expression of *acs* gene in type *a* and not in type *c*. Noteworthy is the fact that the metabolic types found in our complex system (undefined medium, LB) correlate with the results observed in simpler-defined environments, where the presence of stable heterogeneity involving carbohydrate and acetate consumption originated from a unique ancestor has been demonstrated (Helling *et al*., 1987; Rosenzweig *et al*., 1994). Furthermore, overexpression of *acs* gene was found in polymorphic populations growing in simpler environments (Kinnersley *et al*., 2009).

Types *a* and *c* did not reveal significant differences in the transcript levels of the carbohydrate-related genes when grown under H_2O_2 stress. This could be linked to the high impact that the stress factor introduces in their general metabolic activities; as an exception, there was a significant difference in the number of transcripts related to *ptsH* ($P < 0.05$), which suggested a persistent initial response of type *c* under this stress. This coincides nicely with the persistent response observed in the first day of the coexistence experiment in this environment (Fig. 2C).

Distinct transcriptional levels of central metabolism genes during stationary phase

Stationary-phase cells revealed a lower activity of the selected genes compared with cells in exponential phase. Specifically, *galE* transcript levels were significantly higher in type *c* in LB with galactose than in the other two environments ($P < 0.05$) (Fig. 3A). This might be due not only to the preference of this type for galactose but also to the poor performance of type *a* in LB + galactose. The rapid utilization of galactose by type *c* was confirmed by the high levels of aerobic respiration (measured by *cyoA*) observed during growth with galactose. On another notice, *galE* was apparently activated in the later stage of growth of type *a* in LB broth, which is confirmed by the higher levels of *ptsH* in this type during stationary phase. The consumption of trehalose at this point of growth was low for both types *a* and *c*, which corresponds to the early consumption of this sugar during LB growth (Baev *et al*., 2006). While having a faster response during exponential phase (relative to type *a*), type *c* apparently lowers carbohydrate transport at stationary phase when carbohydrates are mostly depleted.

*Differences when dealing with stress (*gadE *and* bolA *genes)*

When cells approach the stationary phase, they start facing several adversities, triggering the activation of specific and general stress response genes. As an *rpoS*-dependent gene, *gadE* is mainly active at stationary phase, although low levels of *rpoS* have also been found in exponential growth phase (Dong and Schellhorn, 2009), implying the activation of *rpoS*-dependent genes. The *gadE* gene product is a regulator of several genes required in the maintenance of pH homeostasis (Hommais *et al*., 2004). We observed a lowered expression of this gene during exponential growth phase and no differences between the two types in LB and/or LB + galactose (Fig. 3B). However, higher and differential levels (P value = 0.054) were observed during early growth in LB + H_2O_2, suggesting an early negative effect of this environment on both types, but a better response of type *a* to this condition, which was maintained until stationary phase. A remarkable increase of the *gadE* mRNA levels was observed in stationary-phase type *a* cells in LB + galactose, possibly linked to the crossed protection response during adverse conditions. During stationary phase, the levels of environmental stress increase and many changes at physiological and genetic level can occur, these results together with the findings during a long stationary-phase culture suggest that type *a* has potentially developed a growth advantage in stationary phase (Zambrano *et al*., 1993).

On the other hand, the *bolA* gene product has been found to respond to several types of stress (osmotic, oxidative, heat, carbon starvation and acid stress), partially independent from *rpoS*. Although there were no significant differences between the types in LB broth and LB + galactose, our results show *bolA* mRNA levels to be higher during the exponential growth phase, surpassing the levels in stationary-phase cells. This response agrees with previous findings, in which *bolA* was induced upon adverse conditions in early growth (Santos *et al*., 1999). mRNA level ($P < 0.05$) was observed during growth with H_2O_2. On the other hand, lower transcript levels of this gene were observed during the last growth phase in all environments; however here, significant differences between types were observed, meaning a differential capacity to deal with stress at this stage of growth.

Altogether, the results reported here suggest the presence of differing roles between these two metabolic types.

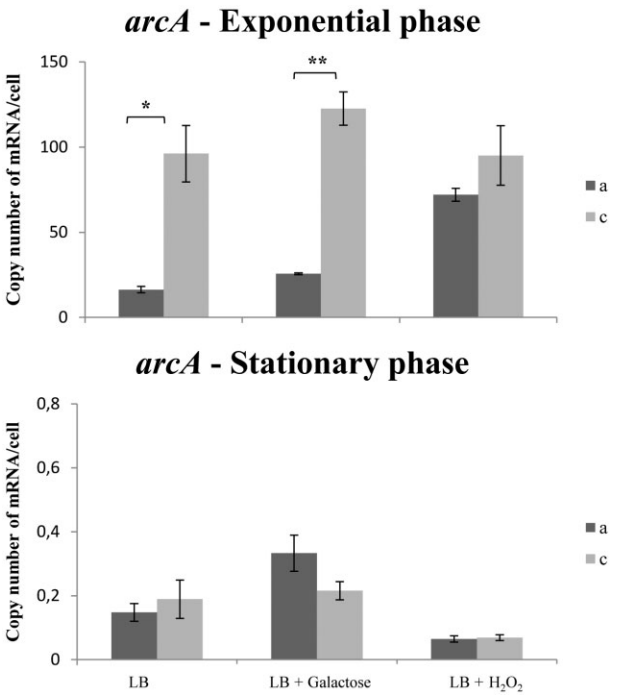

Fig. 4. *arcA* transcriptional assessment. Transcription levels of *arcA* gene.

These roles involved the activation of carbohydrate uptake and consumption by one type as well as an active survival stress response in the other one. The two distinct properties are beneficial to either of the two forms and contribute to maintain a 'balanced' coexistence within the population, in which events of substrate abundance are temporally interspersed with those of stress.

Putative effects of differential arcA *gene mutations*

In the search for additional outstanding phenotypic differences between the two types. We investigated the transcript levels of a major regulator that had been found to have a differential change between the types (Puentes-Téllez *et al.*, 2013). Type *c* colonies carry a mutation in the *arcA* gene (A76V, G- > A), whereas type *a* did not. As a major regulator (controlling the activity of about 168 genes), and making part of a two-component regulatory system, *arcA* is involved in the repression of aerobic metabolism (aerobic respiration, TCA cycle genes) upon anaerobiosis. Under aerobic conditions, *arcA* remains inactive, allowing the normal functioning of aerobic respiration genes (Iuchi and Weiner, 1996). Active *arcA* has also been found to down-regulate and repress *rpoS* in exponential-phase cells (Mika and Hengge, 2005; Hengge-Aronis, 2008). We found a correlation of the effect on *rpoS* related genes with the non-mutant (inactive *arcA*) type *a*, i.e. the transcription levels of genes that are positively regulated by *arcA*, such as those involved in pH homeostasis, *gadE*, *gadA* and *hdeA* (data not shown), are significantly higher in this metabolic type. There might be a correlation with the surprisingly increased levels of *arcA* in type *c* and the repression of *rpoS* genes in this type (Fig. 4). In addition, deletions in *arcA* may be related with hydrogen peroxide sensitive strains (Wong *et al.*, 2007; Gonidakis *et al.*, 2011) and also allow to survive under prolonged starvation (Nystrom *et al.*, 1996; Sevcík *et al.*, 2001). There were remarkable differences in gene expression by type *c* during growth in LB and LB + galactose during exponential phase, and in contrast the expression levels in stationary phase remained equally low (Fig. 4). Moreover, the *cyoA* gene transcription data suggested that the effect of this mutation might have an influence in the respiratory pathways taken by type *c*; a non-mutated *arcA* would be a negative regulator of this gene. Overall, there is a putative effect of this gene on the resulting evolved phenotypes. A direct and indirect effect of *arcA* has been recently found on > 350 genes; however, the full extent of the ArcA regulon remains unclear, preventing a comprehensive understanding of its physiological role (Park *et al.*, 2013).

Conclusions

We unravelled clear phenotypic differences detected among evolved forms in a population of *E. coli* that grew during ~ 1000 generations in LB medium. Adaptive evolution in this organism had, as a consequence, a dichotomy in the growth and survival properties of forms in the resulting population, and the ecological scope of each adaptation influenced the survival capabilities of the type. Distinct responses to stress were observed across the forms, which corresponded to an elevated ability of one particular type to deal with stress at early growth stage. In particular, resistance to oxidative stress was favoured in this form, which might be related to genomic changes affecting specific regulatory characteristics.

Coexistence between the different forms was observed during growth through cycles of 'feast and famine' in LB broth. The stability of coexistence (ratios) was altered when selective pressure was applied that favoured one of the types according to their distinct metabolic and resistance characteristics. Major differences at the gene expression level between the types were observed in the early stages of growth. Such differences are suggestive of niche partitioning and hence enhanced stability in the coexisting population. *Escherichia coli K12* MC1000 evolving in a complex environment like LB apparently copes with the conditions of the environment by two divergent phenotypic responses (that yield coexisting subpopulations: i.e. (i) modulating the metabolism

towards faster feeding of the central pathways and (ii) the favouring of specific properties that allow enhanced survival under stress.

Experimental procedures

Strains

Isolated colonies obtained from an end-point populations of an experimental evolution setup with *E. coli K12* MC1000 were characterized in terms of morphology and metabolic behaviour (Puentes-Téllez *et al.*, 2013; Puentes-Téllez and van Elsas, 2014). We sorted the colonies in three different types: (i) small ('*a*' form), diameter < 1 mm, pronounced centre ('fried egg' shaped), (ii) large/rough/irregular ('*b*' form), diameters > 1 mm and (iii) large/smooth/regular ('*c*' form) with diameters > 1 mm. The population had undergone evolution under aerobic conditions in sequential-batch cultures at 37°C during ~ 1000 generations in LB medium. The ancestral strain was kept frozen at –80°C for further analysis and comparisons.

Assessment of differential survival capabilities

The three forms and the ancestor were exposed separately to oxidative (2.0 mM H_2O_2), osmotic (1.5 M NaCl), heat (49°C) and acidity stresses (pH 3.0) during 4 h. Assessment of survival involved two stages: (i) preparation of the cells (production of the inoculum) and (ii) challenge stage. For the initial stage, cells of each form were grown in triplicate systems until two different growth stages (incubation at 37°C; 200 r.p.m.) in LB medium, i.e. exponentially growing cells (after 5 h, optical density (OD) 600 nm 0.5 ± 0.05) and stationary-phase cells (after 18 h of growth; $OD_{600} > 1.0$). When the desired OD was reached, samples were taken, serving as inocula for the challenge phase of the experiment. For the second stage, inocula were diluted into fresh LB broth to Log 4 cfu ml^{-1}. To reduce effects of the initial growth condition, each stress was applied after 20 min of acclimation (37°C, 200 r.p.m.), time 0. A sample was plated on LB just prior to stress application; these counts were taken as 100% of the population. Population size was then recorded every hour by plating samples on LB medium to obtain the number of viable cells. Percentage survival was determined by dividing the number of cfu ml^{-1} during challenge by the total number of cfu ml^{-1} at time 0 and multiplying by 100. Unless specified, all comparisons of this and further experiments were based on Student's *t*-test.

Coexistence experiment

Two selected 'metabolic types' (*a* and *c*, representing morphologies '*a*' and '*c*' – the latter as a proxy of morphology '*b*' – Puentes-Téllez and van Elsas, 2014) were competed against each other in a crossed experiment. To achieve this, marked strains were obtained by selecting spontaneous mutants to rifampicin (30 μg ml^{-1}) and streptomycin (20 μg ml^{-1}) for each form. The marked strains were analyzed by measuring their fitness after 24 h of growth against their isogenic forms. The relative fitness (*w*) of each mutant relative to their isogenic form was calculated as the ratio of the Malthusian parameters {m = ln[(density at end of competition)/(density at time zero of competition)]} (Lenski *et al.*, 1991). Only mutants with a fitness value equal to 1 ± 0.05 were selected, meaning no difference between resistant and non-resistant types. Fitness was confirmed by measuring in competition each marked strain against its opposite marked form. Coexistence was assessed by growing mixes of the forms (having opposite markers), with daily transfers into fresh media (dilution 1:10 000) after 24 h and during 4 days (three transfers). Growth took place in three different environments, i.e. LB broth, LB supplemented with galactose (0.3%) (LB + galactose) and LB supplemented with a non-lethal concentration of H_2O_2 (0.3 mM) (LB + H_2O_2). Dynamics of the populations was evaluated by plating samples on LB agar with the respective antibiotic, obtaining the population density of each type at exponential (OD_{600} 0.5 ± 0.05) and stationary phase (after 24 h) after each transfer. At the end of competition (d 4), averaged relative fitness was calculated from the ratio between the two types using their Malthusian parameters {ln [density at day 4/ density at time 0 (day 1)]}. After the last sample was taken, cultures were returned to incubation and allowed to continue growing without any transfers for three more days. After this time, samples were plated to assess the percentage of each type in the final population. Individual growth rates (μ) were obtained by growing each type in each environment separately; growth rate was calculated as the slope of the curve during exponential growth phase.

Specific growth rate in supplemented LB broth

Growth rate was evaluated in three different environments. For this, a sample of each type was grown in triplicates in LB broth, LB broth + galactose (0.3%) and LB broth + H_2O_2 (0.3 mM). Growth took place in a microplate reader set at 37°C (VersaMax, Molecular Devices Corp). OD_{600} was recorded every hour. Specific growth rate (μ) was calculated from the steepest slope of the curve against time $\mu = \ln(OD_1/OD_0)/t_1–t_0$.

mRNA isolation

Growth curves of selected colony types were obtained by growing cells at low density in LB broth, LB broth + galactose (0.3%) and LB broth + H_2O_2 (0.3 mM). For this, the optical density of two biological replicates per colony type was recorded at 600 nm using a microplate reader. One ml of cells was harvested at mid-exponential phase (OD_{600} 0.5 ± 0.05) and at stationary phase (OD_{600} 1.1 ± 0.05), number of cfu ml^{-1} in the sample was obtained by plating on LB agar. Pellets, harvested by centrifugation (10.300 × g, 1 min) were immediately used for total RNA isolation with NucleoSpin RNA II isolation kit (Macherey – Nagel, Biokè, Leiden, the Netherlands). Total RNA concentrations and purity were assessed using a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific). Total RNA samples were enriched for mRNA using the MICROBExpress™ Bacterial mRNA Enrichment Kit (Ambion, Austin, TX), which removes the 16S and 23S ribosomal RNAs from the total RNA population. Enriched mRNA populations were reverse-transcribed into cDNA with

the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, USA).

Reverse transcription – quantitative (real time) polymerase chain reaction (PCR)

A total of nine genes were used for amplification with real-time PCR using cDNA as a template. For this, eight primer sets were designed using the default parameters of CLONE MANAGER SUITE (Sci-Ed Software). The sequences of the primer sets used for real-time PCR analysis are shown in Supporting Information Table S1 (Eurogentec, Maastricht, The Netherlands). All real-time PCR runs for each biological replicate were performed in duplicate (total *n* per colony type = 4). The reaction was performed using the SensiFAST™ SYBR Hi-ROX Kit (Bioline) on the ABI Prism 7300 Cycler (Applied Biosystems, Germany). The specificity of the amplification products was confirmed by melting-curve analysis and on 1.5% agarose gels. Standard curves were obtained using serial dilutions of genomic DNA containing a purified sequence (QIAquick PCR purification kit) of each target amplified by PCR (Liss, 2002; Wong and Medrano, 2005). Dilutions ranged from 10^7 to 10^2 gene copy numbers per μl. Data normalization of the results was performed by obtaining the amount of copy numbers per ng of cDNA obtained per sample, then the number of cfu per ml for each sampled time-point was taking into account for calculations (Copy number of RNA/cell; Amount of copy numbers divided by the cells able to form a colony per ml) (Kanno *et al*., 2006).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Experimental setup assessing the phenotypic nature, coexistence and niche differentiation of the emerged forms in the population after experimental evolution.

Fig. S2. Relative fitness (*w*) of metabolic type *a* relative to type *c*. Fitness assessment after competition experiment under three different environments. Competition took place during 4 days with consecutive transfers into fresh medium with or without supplements.

Table S1. Primers used for real-time PCR reactions.

Table S2. Non-consistent mutations in the population. In grey: consistent genes among the three types.

Table S3. Maximal specific growth (μ) in LB broth, LB broth supplemented with galactose 0.3% and LB broth supplemented with non-lethal concentration of H_2O_2 (0.3 mM).