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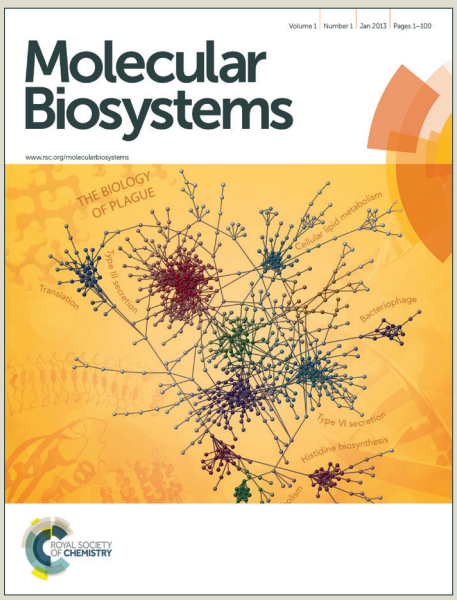

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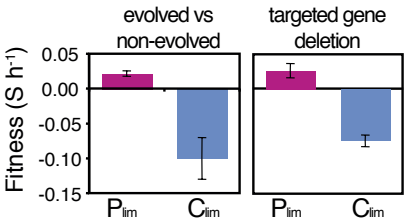
Using full genome sequencing and metabolomics we show that adaptation to chronic nutrient starvation reduces metabolic flexibility in *Escherichia coli*.



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ARTICLE TYPE

A metabolic trade-off between phosphate and glucose utilization in *Escherichia coli*

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Getting the most out of available nutrients is a key challenge that all organisms face. Little is known about how they optimize and balance the simultaneous utilization of multiple elemental resources. We investigated the effects of long-term phosphate limitation on carbon metabolism of the model organism *Escherichia coli* using chemostat cultures. We profiled metabolic changes in the growth medium over time and found evidence for an increase in fermentative metabolism despite the aerobic conditions. Using full-genome sequencing and competition experiments, we found that fitness under phosphate-limiting conditions was reproducibly increased by a mutation preventing flux through succinate in the tricarboxylic acid cycle. In contrast, these mutations reduced competitive ability under carbon limitation, and thus reveal a conflicting metabolic benefit in the role of the TCA cycle in environments limited by inorganic phosphate and glucose.

Free-living organisms like bacteria face the challenge of simultaneously obtaining all elements they require for growth in the right proportions. Based on metabolic network studies, it has been suggested that metabolic evolution is shaped by two opposing needs; to maintain high metabolic flexibility between conditions, and to achieve near-optimal growth in one condition.[1] We were interested in the effect of continued, long-term nutrient limitation on this evolutionary equilibrium and hypothesised that chronic starvation for one nutrient was likely to tip metabolism in favour of growth specialization, or, in other words, give rise to mutants that had lost some of their metabolic flexibility, but would show increased fitness in the limiting conditions.

The regulatory and metabolic systems controlling uptake and assimilation of at least some different nutrients are interlinked, co-dependent and complex, for example for carbon and nitrogen sources.[2,3] On the other hand, we know little of the interplay between carbon and phosphorus assimilation, and how this influences bacterial fitness in different environments. To test whether selection for specialization alters metabolic flexibility, we grew *Escherichia coli* in low concentrations of inorganic phosphate (Pi), an important nutrient that restricts microbial growth in many freshwater and marine environments.[4] We monitored bacterial growth in two independent populations for 30 days, a time-frame sufficient for mutational shifts in the population,[5] and measured metabolic changes in the media using NMR-based metabolic profiling (see supplementary file for details of all methods); exometabolome analysis (or metabolic footprinting) can be biochemically revealing, and extracellular metabolite concentrations are generally a result of metabolic regulation rather than cell lysis.[6] We observed a similar and stable biomass trajectory over this period (Fig. 1a), while residual glucose decreased markedly after 12 days (Fig 1b), with a concomitant increase in formate, lactate and succinate accumulation in both populations following this point, indicating an increase in mixed acid fermentation products (Figure 1). Concentrations of these acids peaked and began to decrease after 25 days, suggesting further sweeps by sub-populations altered in glucose metabolism. Additional complexity was shown by acetate (Fig 1f), a common overflow metabolite in the presence of excess glucose, as it increased in concentration independently of formate/lactate/succinate (Figure 1; Supplementary Figures S1 and S2, Supplementary Tables S1 and S2).

We employed a three-step process to identify the genes controlling this metabolic link between Pi and central carbon metabolism. We first screened isolates sampled at day 19 (Supplementary Table S3 for details) for increased fermentative metabolism by introducing them into new chemostats and running targeted assays for these fermentation products (Figure S). We then sequenced a mixed acid producing strain (BW6043) and a non-producing strain (BW6041) and compared the full genome sequences to the ancestral strain as before.[7] We found multiple mutations with respect to wild-type in both strains (Supplementary Table S4) including a mutation in rpoS, which is a known feature of adaptation to nutrient limited environments.[5] The only differences between the strains were two intergenic mutations in the *sucABCD* operon, between sucB and sucC. Finally, we measured the competitive fitness of BW6043 and BW6041 against each other in a chemostat environment and found that the *sucBC* mutation in BW6043 provided a positive fitness advantage over BW6041 under Pi-limitation (Fig 3).

Based on the structure of the *sucABCD* operon, we predicted that the intergenic sucBC mutation would affect sucCD but not *sucAB* transcription. To test this, we tested a lab-generated sucC knockout for increased fermentation products and altered fitness. As expected, the *sucC* knockout strain (BW3789) produced increased amounts of lactate and succinate (even more than BW6043; Figure 2). The knockout had higher fitness under Pi

limitation in competition experiments against a *sucC*+ strain, demonstrating that disruption of *sucCD* transcription is sufficient to provide a fitness benefit under Pi limiting conditions (Figure 3). Our results confirm previous reports of increased fermentative metabolism in *E. coli* under P limitation, but without linking this to a specific enzyme.[8,9]

What is the physiological role of the enzymes encoded by the *sucABCD* operon? *sucAB* and *sucCD* encode two distinct, mutually essential TCA cycle enzymes.[10] SucAB is part of the α-ketoglutarate dehydrogenase complex, catalysing the irreversible conversion of α-ketoglutarate to succinyl-CoA and CO2, while *sucCD* encodes succinyl coenzyme A synthetase (SCS), which reversibly catalyses the conversion of succinyl-CoA to succinate. Cells lacking SCS do not have a fully functional oxidative TCA cycle, forcing the organism into 'anaerobic' mode. Under anaerobic conditions, *E. coli* operates a branched TCA cycle, with an oxidative branch from citrate to α-ketoglutarate and a reductive branch, from oxaloacetate, fed from PEP via carboxykinase.[11] These conditions are associated with flux into mixed-acid fermentation, but, importantly, also allow higher maximal glucose uptake rates).[12] The mutational sweep of the populations by the *sucBC* mutant also further increased glucose uptake by around threefold (9 mM residual glucose to less than 5 mM, with 11 mM glucose originally present in the medium). We propose that the higher rate of consumption of a non-limiting nutrient (glucose) increases the proportion of Pi assimilated by substrate-level phosphorylation during glycolysis, and reduces the proportion assimilated by oxidative phosphorylation. We predicted that this mutational optimisation of growth under Pi limitation would diminish the organism's metabolic flexibility. Supporting this prediction, strains with a disrupted TCA cycle exhibited a decreased fitness under the reverse nutrient limitation, i.e. in glucose-limited conditions with excess Pi, demonstrating the antagonistic pleiotropy of the growth optimisation to one condition.

Conclusions

In summary, our laboratory evolution provides evidence that phenotypic plasticity is reduced under prolonged P limitation in favour of promoting substrate optimality for this condition. This suggests that P and C assimilation are linked in a metabolic trade-off. Fully integrated metabolic models of bacterial metabolism will need to incorporate such links in balancing resource use between carbon, nitrogen, and phosphorus sources in bacteria, as partially shown in ref 13.

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The authors declare no competing financial interests.

Supplementary information is available for this article at the RSC website.

Notes and references

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‡ Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

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Figure legends

Figure 1. Population density and extracellular metabolites in two long-term phosphate-limited chemostat cultures. *E. coli* strain BW2952 was grown at a dilution rate of 0.1 per hour. A: Optical density, B: Glucose, C: Succinate, D: Formate, E: Lactate, F: Acetate.

Figure 2. Accumulation of extracellular lactate (A) and succinate (B) in wild-type (BW2952), evolved isolates (BW6041 and BW6043) and a strain with sucC deletion (BW3789). Cultures were grown on phosphate limited T-salts minimal medium (Wang et al. 2009) supplemented with 35 μM KH2PO4 and 0.2% w/v D-glucose in

chemostats at a dilution rate of 0.1 per hour. Samples were withdrawn from the 72-old steady-state cultures and analysed by HLPC. The compounds were identified by comparison with authentic standards. The integrated peak areas for lactate and succinate were used to estimate the percentage of these compounds in the total peak area of all detected compounds. Data presented are average of two independent biological replicates. See Table S1 for details of strains used in this study.

Figure 3. Competitive fitness of strains under Pi-limited and Pi-excess-Glc-limited environments. (A) Fitness of BW6043 against the coexisting strain BW4041 that differ only in three mutations; two of them are located in intergenic region between *sucB* and *sucC* (Table 2). (B) Fitness of BW3789 ($rpoS^{-}sucC^{-}$ strain) against the BW5318 ($rpoS^{-}sucC^{+}$ strain). (C) Fitness of BW6043 against BW5318. All competitions were performed in a steady-state chemostat at a dilution rate of 0.1 per hour. (D) Summary of the physiological changes between ancestral and evolved strains. For all competitions, each plotted value represents the mean ± standard deviation from three to four independent experiments.