Unraveling the impact of compensatory evolution on metabolic divergence

Background and aims

The constructive role of deleterious mutations in evolutionary innovations is generally disregarded. In general, mutations that impair gene or gene product function are harmful to the organism's fitness, therefore are thought to be eliminated from populations by natural selection (Charlesworth, 2012). However, they can reach high frequencies in populations when they hitchhike with beneficial mutations (Lang *et al*, 2013). In such cases, compensatory evolution may occur, where other genetic changes elsewhere in the genome can offset the negative effects of the deleterious mutation and restore the organism's fitness (Elena & Lenski, 2003). In this work, we address a long-standing debate on the role of non-adaptive mutations in generating evolutionary novelties. Here, we propose that compensatory evolution following the fixation of deleterious loss-of-function mutations initiates major changes in molecular and phenotypic traits without direct selection on them. By collecting phenomic and transcriptomic data we aim to systematically study the constructive role of compensatory evolution in microbial evolution. We investigate this question in two separate research projects; i) we study the effect of compensatory evolution on the emergence of new morphologies by focusing on Saccharomyces cerevisiae, ii) we study how compensatory evolution-driven metabolic alterations contribute to phenotypic innovations.

Results

During the project period, we have made significant progress toward the project's goals and have prepared two manuscripts, one of which has already been published in a leading journal.

We aimed to follow the project proposal, although we reconsidered some questions to be investigated, which caused some alterations compared to the original research plan: we expanded the scope of our study to systematically examine the constructive role of compensatory evolution in microbial evolution. We investigated this question in two separate research projects; i) we studied the effect of compensatory evolution on the emergence of new morphologies by focusing

on Saccharomyces cerevisiae, which is amenable to high-throughput morphology analysis (see #1), ii) we study how compensatory evolution-driven metabolic alterations contribute to phenotypic innovations in E. coli, which is a well-established model system to study the evolution of metabolic novelties (see #2).

Our main results are as follows:

1) Gene loss and compensatory mutations promote phenotypic evolution in yeasts

We studied the effect of compensatory evolution on the emergence of new morphologies by focusing on Saccharomyces cerevisiae. Specifically, we investigated the hypothesis that evolutionary compensation for loss-of-function mutations does not fully restore cellular physiology, but rather leads to new cellular phenotypes. Our analysis of a systematic collection of fitness-compensated yeast strains generated earlier in our laboratory (Szamecz *et al*, 2014) revealed that yeasts with gene losses can rapidly and frequently evolve new morphologies, even multicellular ones, such as invasive growth, multicellular aggregation and biofilm formation, which can be both ecologically and clinically relevant. In this study, we provide the first evidence to support the hypothesis that gene loss and compensatory mutations are driving forces of phenotypic innovation. As a result, our discovery challenges the commonly held belief that loss-of-function mutations have no relevance to adaptive evolution. Our results were recently published in Nature Ecology and Evolution (IF=15.46). In addition to receiving considerable attention on social media (Twitter), the paper has also been featured in an editorial piece in Nature Reviews Genetics.

2) Compensatory evolution-driven metabolic alterations drive pre-adaptation to new nutrients via transcriptional rewiring

To systematically study whether new nutrient-utilization phenotypes can evolve through compensatory evolution, we took advantage of a set of laboratory-evolved single-gene deletion *E. coli* strains derived from a previous experimental evolution study (Blank *et al*, 2014). In a

nutshell, 22 ancestor single gene knock-out *E. coli* strains were subjected to laboratory evolution in 5 parallel replicates in minimal medium, which allowed growth rescue via compensatory genetic changes. In total, laboratory evolution yielded 68 independently evolved lines that recovered growth in minimal medium. This strain set presented a unique opportunity to study the effect of compensatory evolution via metabolomics approaches for several reasons: (1) all strains lack one of 22 metabolic genes that are essential for growth in minimal medium (Blank *et al*, 2014), (2) because essential gene functions were lost, functional innovations had to arise to rescue the growth phenotype, (3) the list of deleted genes encompasses diverse metabolic functions, (4) many high-growth-rate compensated lines emerged during compensatory evolution (i.e. close to wild-type fitness), (5) parallel evolved replicate lines allow us to study alternative evolutionary scenarios, (6) the mutations accumulated during compensatory evolution were identified by whole-genome sequencing, (7) only a limited number of mutations accumulated, thus understanding the genetic basis of compensatory mechanisms is feasible.

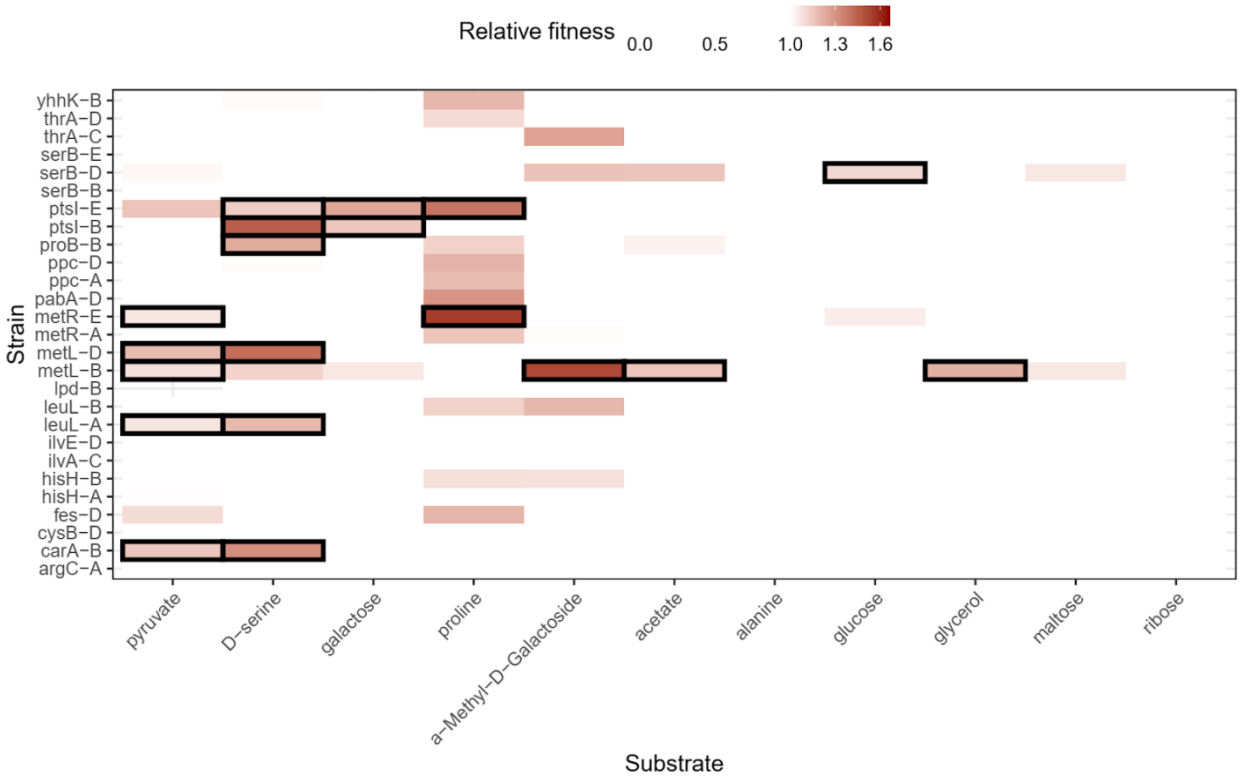
Our investigations were organized around three primary questions:

- **Does compensatory evolution lead to new alternative metabolic configurations?**
- **Can gene expression changes promoted by compensatory evolution contribute to fitness gain in novel environments?**
- **Are there general rules in the molecular mechanisms underlying fitness gains in novel environments?**

Does compensatory evolution lead to new alternative metabolic configurations?

Evolutionary innovations are frequently defined as qualitatively new and adaptive traits of an organism (Wagner, 2011). The classical route to innovate a new growth phenotype is by adapting to the new environmental challenge. Here we asked whether such 'innovations' can emerge as a byproduct of compensatory adaptation, which could be a real possibility if entirely new metabolic configurations evolve to rescue the loss of essential enzymes. We hypothesize that alternative metabolic configurations can promote adaptation to a novel environment, either by quantitatively increasing the growth rate above the wild-type (fitness gain) or by enabling

growth in an environment where the wild-type is unable to grow (innovation). To test whether new nutrient-utilization phenotypes can evolve through compensatory evolution, we initiated a phenotypic screen across 10 different carbon sources. We used a high-throughput measurement protocol to determine the rate of compensation in each of the 68 evolved lines. An engineered BW25113 - in which a kanamycin resistance cassette was integrated into the IS150 element - was used to determine the reference wild-type fitness level. We chose 26 compensated strains above 0.8 relative growth rate for further investigation. Wild-type and evolved populations were subjected to high-throughput fitness measurements by monitoring growth rates in liquid culture across 10 different carbon sources. Growth rates of evolved lines were compared to wild-type to look for significant fitness gains in a given environment (Wilcoxon test, 5% false discovery rate, Figure 1A) We found significant fitness gains (maximum 60% relative gain), in about third of the strains and half of the carbon sources, amounting to 7% of all strain-carbon source combinations. One may argue, that initial gene deletions alone could result in improved nutrient utilization. Although this scenario is not consistent with the several cases of fitness differences we see between parallel compensated strains stemming from the same ancestral deletion strain. To investigate this question further, we remeasured the growth rate of our significant hits together with their ancestral deletion strain. We found that none of the ancestral strains grew significantly better than the wild-type suggesting that in general, not the deletions alone are responsible for the fitness gain in alternative carbon sources, but compensatory mutations also contribute (Figure 1B.).


Relative fitness
0.0
0.5
1.0
1.3
1.6
Strain
yhhK−B
thrA−D
thrA−C
serB−E
serB−D
serB−B
ptsI−E
ptsI−B
proB−B
ppc−D
ppc−A
pabA−D
metR−E
metR−A
metL−D
metL−B
lpd−B
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ilvE−D
ilvA−C
hisH−B
hisH−A
fes−D
cysB−D
carA−B
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maltose
ribose
Substrate

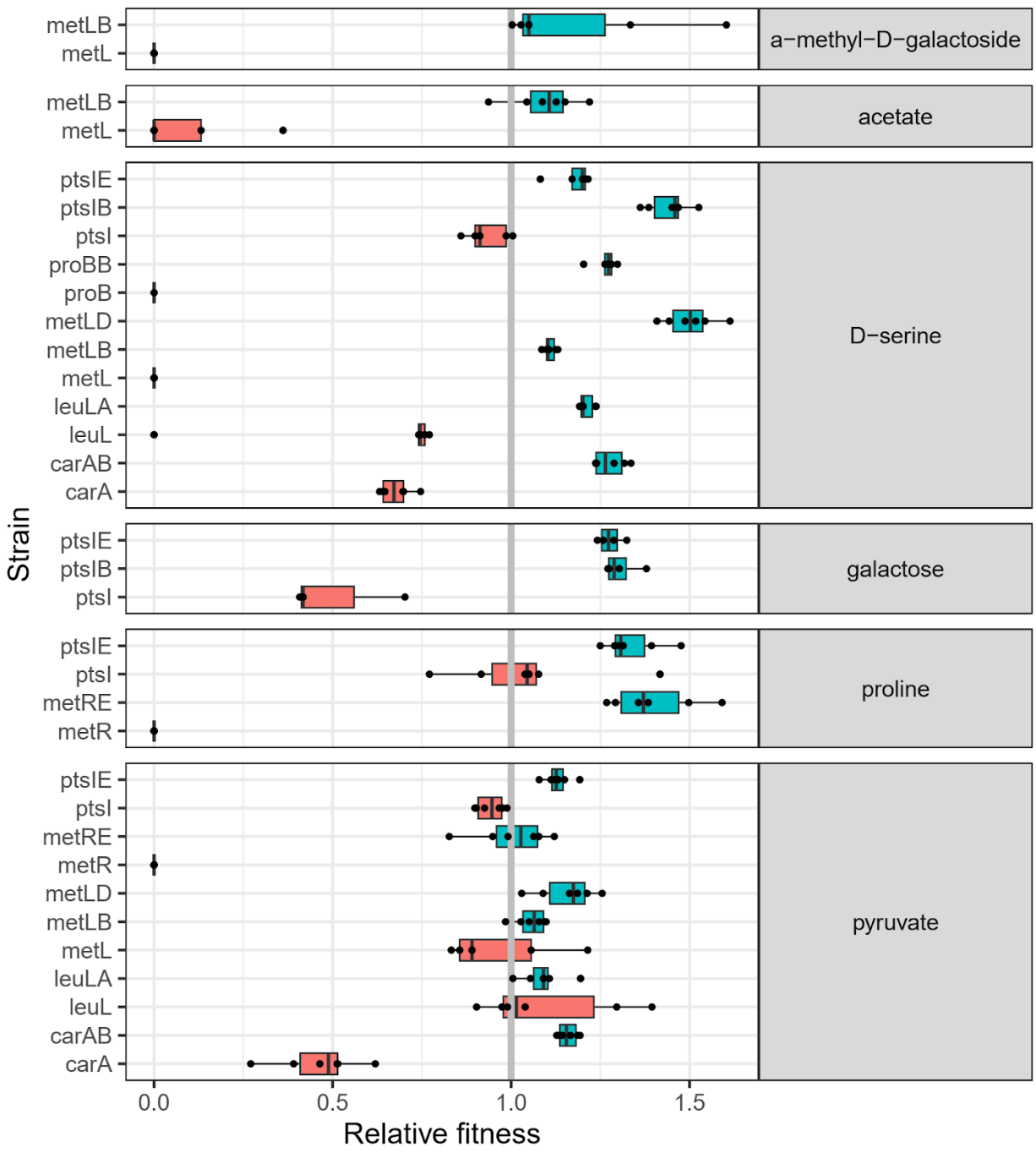


Figure 1A.) Heatmap indicating the relative growth rate of compensated strains in different carbon sources. Only relative growth rates above 1 are coloured for better visibility of the increases in growth rate. Black outlines indicate cases having statistically significantly higher than wild-type growth rates. **B.)** Boxplots indicating the relative growth rate of compensated strains and their ancestors on different carbon sources. Only those compensated strains are shown which displayed fitness increase in our previous measurements.

In sum, we found that in several deletion strains, compensatory evolution can substantially increase growth rate above the wild-type in diverse alternative carbon sources, thus it can frequently promote adaptation to novel environments as a side-effect.

Can gene expression changes promoted by compensatory evolution contribute to fitness gain in novel environments?

Our previous work demonstrated that new gene expression states evolved due to gene deletion followed by compensatory evolution (Szamecz *et al*, 2014). However, it is unknown how transcriptomic changes contribute to the formation of alternative metabolic configurations, which then can promote adaptation to a novel environment. Here we carried out whole transcriptome RNA sequencing and analysis for 10 compensated strains stemming from 6 single-gene deletant ancestors (glucose minimal media) to find mechanistic explanations for the compensated strains' fitness advantages in different carbon sources.

Our results showed that the degree of transcriptional rewiring across compensated strains was substantial across compensated strains, with significant transcript level changes ranging from 164-585 (FDR=0.05, absolute fold-change > 2), meaning that the compensated strains did not restore their original expression levels, as it was reported in earlier studies (Szamecz *et al*, 2014; McCloskey *et al*, 2018b). To gain an overview of the strains' transcriptome profiles we looked at the expression changes of statistically independent transcriptional modules, by which we identified both general and strain-specific transcriptomic changes (Figure 2.). General transcriptomic changes can be related to medium adaptation. For example upregulation of motility genes or a set of downregulated genes related to pH and membrane homeostasis, are also frequently seen in medium adapted strains.

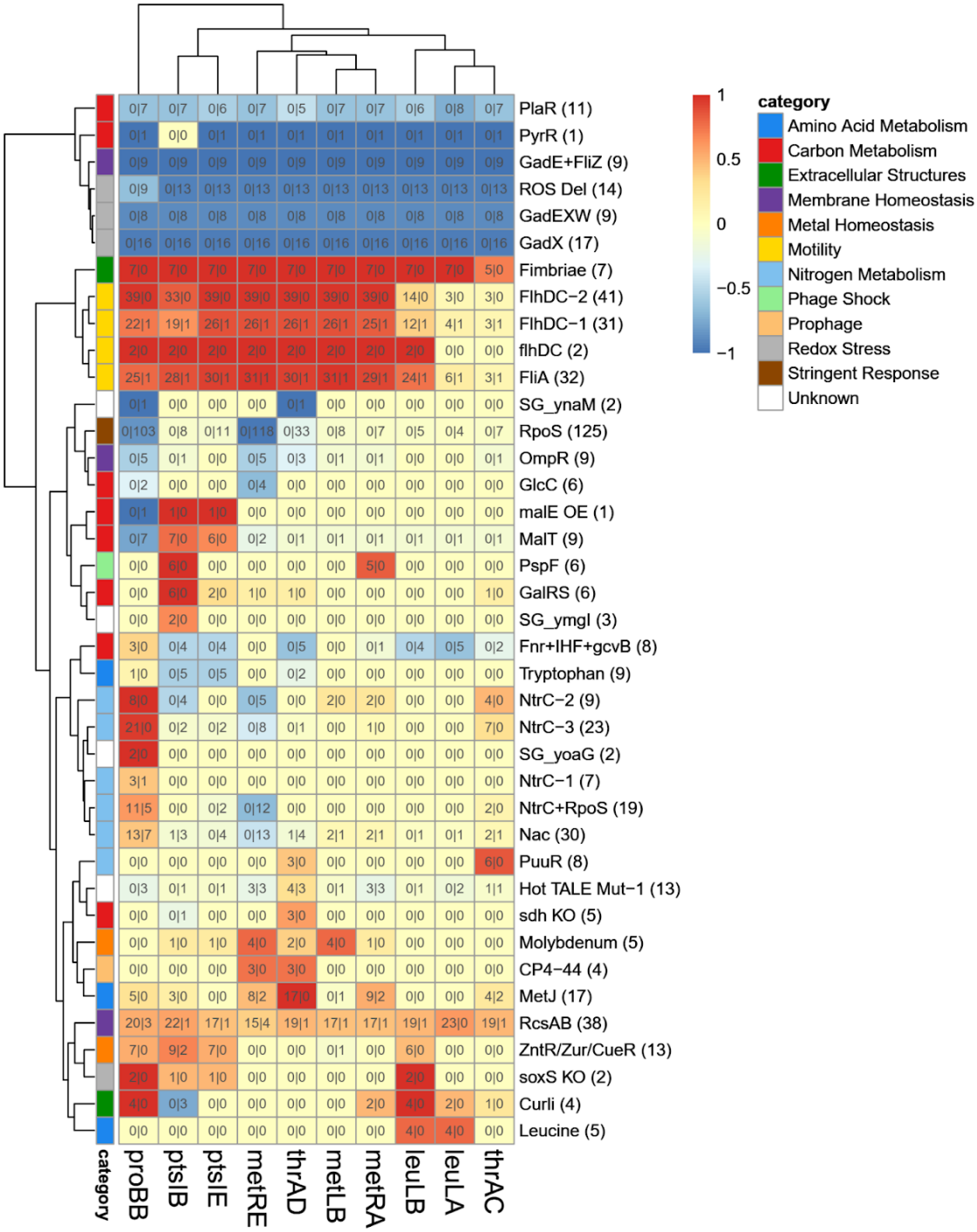


Figure 2) Heatmap representing transcriptome profile changes in 10 compensated strains (from 6 deletions) compared to the wild-type in glucose minimal medium. On the y-axis, the names of transcriptional modules (iModulons) and the number of genes belonging to the modules are indicated. Numbers within cells represent the number of genes active in a given module being significantly up- or downregulated in a given strain (|fold-change|>2, FDR = 0.05). The proportion of genes within a module being up or downregulated are indicated with red

and blue colours, respectively. In the few cases where genes of a module are not exclusively up- or downregulated, the colours indicate either the proportion of up- or downregulated genes having the higher value. Only those modules are shown, in which at least half of the genes are changing their expression level in at least one strain. Transcriptional modules and their functional categories were downloaded from the Precise 2.0 database (Lamoureux *et al*, 2021).

Interestingly, the number of strain-specific transcript level changes, attributable to the specific deletions and compensatory mutations is still substantial. These involve changes in functionally diverse transcriptional modules with diverse functional categories not always closely linked to the deleted gene's function, including the general stress response (RpoS), phage shock protein system (PspF), etc.

We hypothesize that these transcript level changes can sometimes, as a side-effect increase growth in alternative carbon sources. For example, altering a given gene's transcript level can be both compensatory and as a side-effect, can also be useful when growing in a different carbon source. Alternatively, a compensatory mutation affecting a transcription factor can affect many genes in one or more transcriptional modules, not necessarily related to the compensation, but increasing the chance of pleiotropic side effects. Thus, the modular hierarchy of the transcript-level changes can also facilitate preadaptation to different environments.

Are there general rules in the molecular mechanisms underlying fitness gains in novel environments?

We integrated genomic, transcriptomic and phenotypic data to categorize molecular mechanisms responsible for fitness gain in novel environments. We examined three representative case studies to reach our conclusions:

- ***Fitness gain on D-serine can be the byproduct of the pleiotropic effect of a transcription factor mutation on the regulatory network***

The compensated strain, proB-B, grows better in D-serine than the wild-type. ProB is a glutamate kinase, catalyzing the first step of proline biosynthesis. Transcriptome analysis reveals that a D-serine/alanine/glycine transporter, cycA is exclusively upregulated (~ 4x) in proB-B

among the 9 examined strains. This D-serine transporter upregulation provides a strain-specific mechanism to increase growth on D-serine. This upregulation can be linked to mutations in the transcriptional regulatory network indirectly affecting cycA. The upregulation of cycA can be explained by the upregulation of its transcriptional activator nac (nitrogen assimilation control protein, ~ 64x). The transcription of nac can be in turn activated by another transcriptional regulator, NtrC, encoded by glnG. Based on gene expression profiling, NtrC encoding gene glnG is significantly overexpressed in proBB (~ 3x). GlnG is part of the glnALG operon which harbors two mutations, one SNP in an internal promoter upstream glnL (-9, C->A) and one in glnA (D154N), which might affect their overexpression. Mutations in glnA have been repeatedly found in parallel lines of proB deletion strains and assumed to be compensatory by reducing its activity thus creating a larger glutamate pool thereby increasing the flux in the direction of an alternative enzyme with a low-level underground activity, to compensate for proB loss (Guzmán *et al*, 2018). NtrC and Nac control transcriptional response to nitrogen starvation, regulating many genes involved in nitrogen metabolism and it is not yet clear how upregulating either might compensate for proB loss.

Based on our results we can hypothesize that the upregulation of cycA is a non-compensatory byproduct of the activation of NtrC, which can have a compensatory effect by regulating other, compensatory gene(s) (Figure 3.).

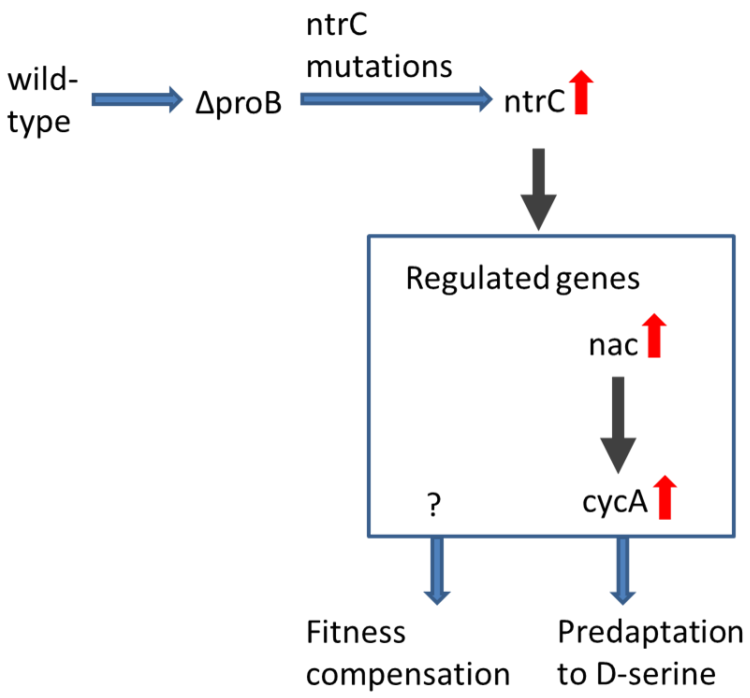


Figure 3. Our model of evolving higher growth on D-serine as a side-effect of compensatory mutations affecting a transcription factor also indirectly upregulating a D-serine transporter.

Taken together, these results suggest a general mechanism, which can lead to preadaptation to a novel environment. The widespread pleiotropic effects of a mutation in a transcription factor acting on the higher levels of the regulatory hierarchy can provide opportunities for preadaptation to novel environments by affecting many non-compensating genes in a transcription module, which might be beneficial under alternative conditions.

• ***Fitness gain on galactose is a side-effect of compensatory upregulation of a substrate-ambiguous galactose transporter***

On galactose carbon source, two compensated strains from the same deletion, ptsI-B and ptsI-E grew significantly better than the wild-type (relative growth rates 1.17 and 1.25, respectively, Figure 1.). PtsI encodes for PTS enzyme I, a constituent of the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS), which is the main system for the import and phosphorylation of multiple carbohydrates, including glucose. In ptsI-B, the gene galR, a repressor of galactose transfer and catabolism genes harbored a nonsynonymous (V->A)

substitution in its DNA binding region. We hypothesized that this mutation caused the derepression of its regulated genes, which could be responsible for the increased growth in galactose-containing medium. Our hypothesis is supported by systematic fitness screens of single gene deletion strains showing that deleting galR increases both growth rate and competitive fitness on galactose carbon-source (Tong *et al*, 2020; Price *et al*, 2018). To further test our hypothesis, we examined the transcript level changes in the gal genes regulated by galR. Our transcriptome measurements confirmed that in the ptsI-B strain both the galP galactose transporter and galETKM operon, encoding genes involved in galactose degradation, are significantly upregulated. Furthermore, galP is also upregulated in ptsi-E to a comparatively high degree (11.8x and 10x overexpression in ptsI-B and ptsI-E, respectively), while no gal genes were upregulated in any of the other 8 compensated strains with transcriptome data. In the original study of the compensated strains (Blank et al. 2014) no such mutation was listed in ptsI-E which could explain the overexpression of galP. However, we found that the DNA sequence coverage for the promoter region of galP was missing, therefore we PCR-sequenced this region to look for potential mutations explaining the overexpression of galP. Indeed we found an SNP (T->A, -189) which could potentially affect the regulation of galP. The very same mutation was found in a former lab-evolution experiment, where the deletion of ptsI and two functionally linked genes (crr and ptsH) together was compensated in glucose medium (McCloskey *et al*, 2018a). Furthermore, galP was found to be more highly overexpressed in that strain than in parallel compensated strains lacking the galP mutation (McCloskey *et al*, 2018a). These results suggest that this galP mutation can indeed have a compensatory effect by overexpressing galP and galP overexpression can increase growth on glucose and galactose in both ptsI-B and ptsI-E (Figure 4.).

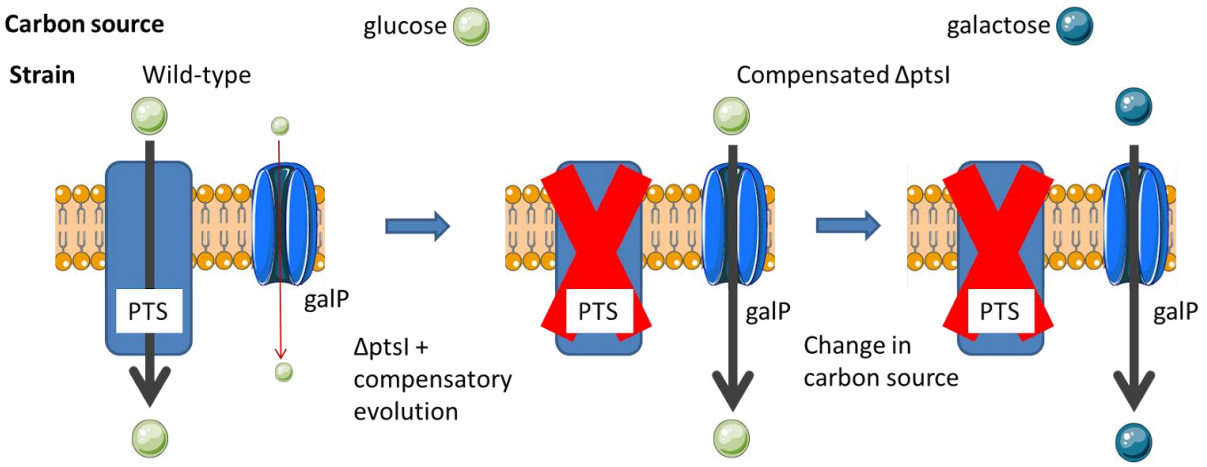


Figure 4. Our model of evolving higher growth on galactose as a side-effect of ptsI deletion and compensatory evolution affecting galP expression to restore glucose transport.

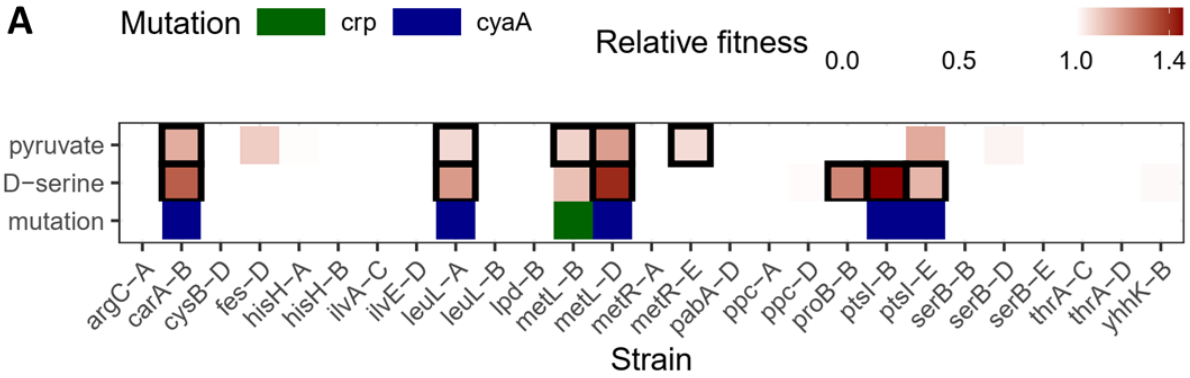
In sum, it has been previously found that overexpression of transporters showing substrate ambiguity can be a general mechanism to suppress the deleterious effect of gene deletions (Patrick et al, 2007). Our results also suggest that upregulating such a multifunctional transporter during compensatory evolution can also be a mechanism to provide fitness advantage in a different carbon source.

- ***Fitness gain on multiple carbon sources can be achieved by general compensatory mutations affecting catabolite repression***

On D-serine carbon source, 6 strains of 4 deletion backgrounds were able to grow significantly better than the wild-type, beside the already discussed proB-B, and 5 of these also outgrow the wild-type on pyruvate (Figure 5A). The high number of strains with increased fitness suggests a general compensatory mechanism across deletion strains, while the overlap between the two carbon sources also suggests a common mechanism for D-serine and pyruvate. Indeed, the two carbon sources are close to each other in the metabolic network: D-serine is degraded to pyruvate in only one enzymatic and two following spontaneous reaction steps.

We hypothesize that this general compensation mechanism works by repressing the costly expression of genes upregulated in slow-growing strains via the CRP-cAMP system (CRP: cAMP receptor protein). This hypothesis is suggested by the mutation profiles, since almost all

of the strains growing faster than wild-type harbor loss-of-function mutations affecting the CRP-cAMP system. To further test our hypothesis we introduced the cyaA mutation found in the carA-B strain into the wild-type (WT+cyaA) the carA deletion strain (carA+cyaA) and in a carA deletion strains where the compensatory gene carB is overexpressed on a multicopy plasmid. In agreement with our hypothesis, WT+cyaA significantly increased its growth rate compared to the wild-type on D-serine. Surprisingly, we couldn't see cyaA mutation increasing growth on carA deletion background (carA+cyaA), only when overexpressing the compensatory gene carB. CarB overexpression in the carA deletion strain only slightly increases growth in D-serine (carA+carB, Figure 5), while together with cyaA mutation it reaches the growth of the carA-B compensated strain, suggesting synergistic epistasis between cyaA and carB mutations. Thus, our experiments support that deactivating the CRP-cAMP system can be beneficial in D-serine for a partially compensated deletion strain, and it can also result in faster than wild-type growth.



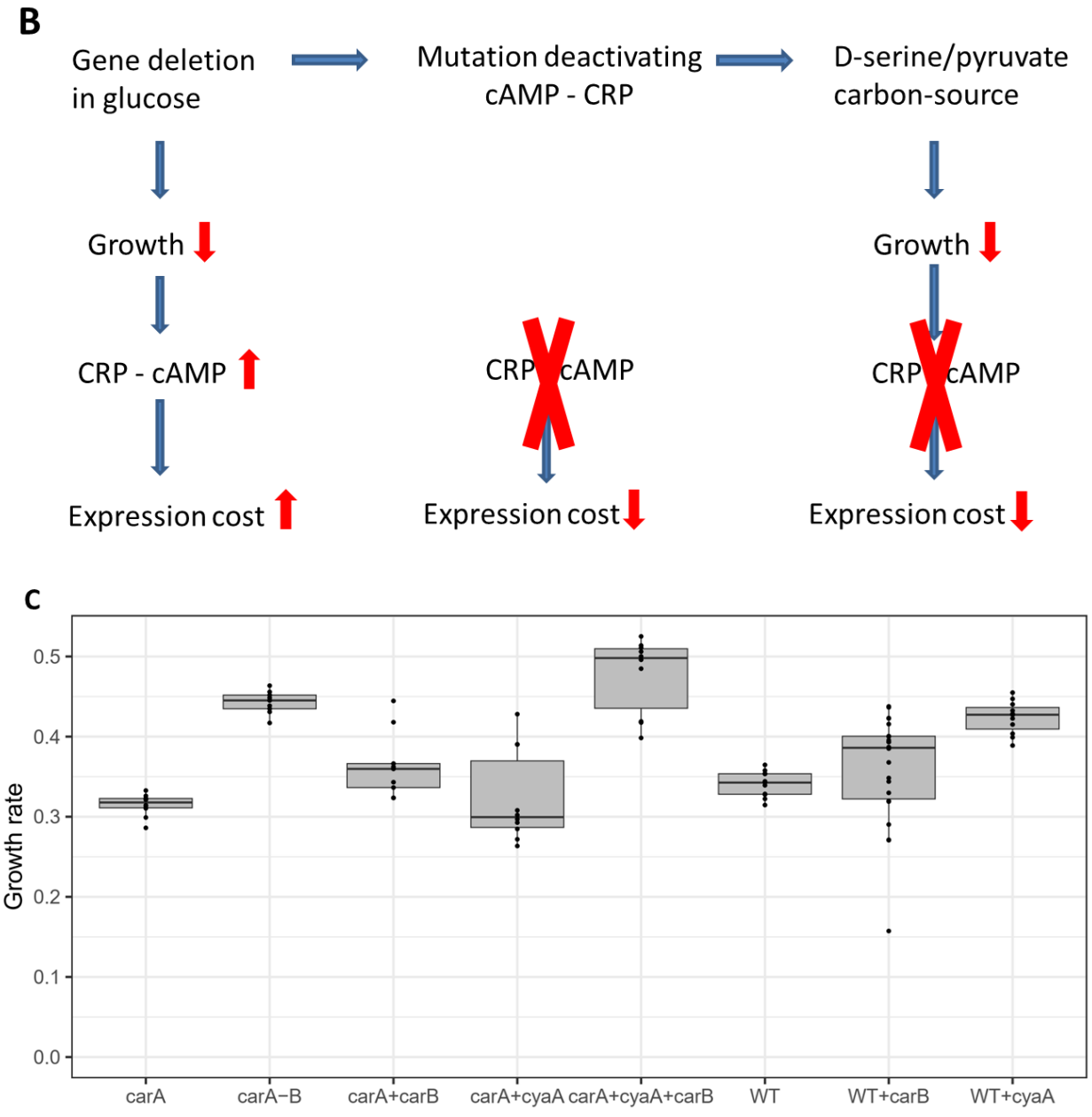


Figure 5: A) Heatmap indicating compensated strains having cAMP-crp related mutations (either in the genes crp or cyaA) and higher than wild-type growth rates of in pyruvate and D-serine. Black outlines indicate cases having statistically significantly higher than wild-type growth rates. **B)** Our model of evolving higher growth in D-serine/pyruvate compared to wild-type. Deleterious gene deletions by decreasing growth induce the CRP-cAMP system which incur a further fitness cost. This cost is abolished by mutations deactivating the CRP-cAMP system during compensatory evolution. Alternative carbon sources result in slower growth than glucose, but the compensated strains cannot activate the costly CRP-cAMP system, therefore they can gain a fitness advantage over the wild-type strain with intact CRP-cAMP system. **C)** Boxplots indicating the growth rates measured on D-serine

for carA ancestral and compensated deletion strains and reconstructed strains with the carB and cyaA compensatory mutations introduced to the WT and carA deletion background.

Taken together, the cAMP-CRP system can be activated by slow growth, and its disruption can be generally beneficial under slow-growth conditions, caused by either a deleterious mutation or an altered carbon source. Such a disruptive mutation can be compensatory in slow-growing deletants, and as a side-effect, also beneficial in multiple alternative carbon sources, where coli grows slowly Thus, the same general mechanism can provide preadaptation for multiple deletion strains in multiple environments. Preadaptation to the same environment can also be achieved by both general and strain-specific mechanisms.

Conclusions

We organized our investigations around three primary questions, which all have been answered during the project period. With that, the major scientific goals presented in the project proposal have been accomplished. Moreover, we expanded the scope of our study to systematically examine the constructive role of compensatory evolution in the evolution of microbes.

Here I will summarize our findings related to the project proposal:

We identified several deletion strains in which compensatory evolution substantially increased growth rate above the wild-type in diverse alternative carbon sources. Considering that independently evolved populations showed fitness variation across environments, our results suggest that the accumulation of adaptive mutations during compensatory evolution generated hidden differences in their metabolic functions, which were then uncovered upon exposure to a novel environment. Taken together, compensatory evolution-driven metabolic alterations promoted adaptation to novel environments as a side effect. This presents a radically new evolutionary scenario as it works through pre-adaptation in a constant environment.

We integrated genomic, transcriptomic and phenotypic data to identify different forms of molecular preadaptation: alteration in specific functions, global network changes, as well as activation of a transcriptional module as a byproduct could also lead to environmental fitness gains. These findings suggest that new adaptive traits emerge through gene loss and subsequent compensatory adaptation, without direct selection on them.

Although our results provide significant evidence for the constructive role of compensatory evolution in adaptive evolutionary processes, this study has to be seen in the light of some limitations. Here we primarily aimed to examine how compensatory evolution can alter the metabolic configuration of evolved bacterial lines. Thus it was obvious to screen for environments where changes in metabolism are most easily identified. For this reason, we used various carbon sources as screening conditions. The impact of altered metabolism on adaptation to other stressors, such as osmotic stress, pH, antibiotics, etc. was not examined.

Taken together, our results showed how gene deletions followed by compensatory mutations can provide the raw material for the evolution of novel traits that allow a population to better exploit new ecological niches. Thus, compensatory evolution plays an important role in the adaptive process, enabling populations to persist and diversify in changing environments. In this context, understanding the genetic basis of compensatory evolution is crucial for gaining insights into how organisms adapt to changing environments and how they may respond to future challenges.

Despite the successful scientific efforts, unfortunately the Covid-19 pandemic had an impact on the final accomplishment of the project. The laboratory work was encumbered by the pandemic situation; shiftwork regulations, as well as shipping delays in laboratory consumables and supplies hindered the phenotypic screens and molecular cloning. However, our second manuscript will be published in a Q1-D1 journal soon with my first authorship (The manuscript is attached to the report via Google Drive).

Publication and presentation activity

Published papers:

Károly Kovács, Zoltán Farkas, Djordje Bajić, Dorottya Kalapis, Andreea Daraba, Karola Almási, Bálint Kintses, Zoltán Bódi, Richard A Notebaart, Juan F Poyatos, Patrick Kemmeren, Frank C P Holstege, Csaba Pál, Balázs Papp, Suboptimal Global Transcriptional Response Increases the Harmful Effects of Loss-of-Function Mutations, Molecular Biology and Evolution, Volume 38, Issue 3, March 2021, Pages 1137–1150, https://doi.org/10.1093/molbev/msaa280

Zoltán Farkas, Károly Kovács, Zsuzsa Sarkadi, Dorottya Kalapis, Gergely Fekete, Fanni Birtyik, Ferhan Ayaydin, Csaba Molnár, Péter Horváth, Csaba Pál & Balázs Papp: Gene loss and compensatory evolution promotes the emergence of morphological novelties in budding yeast. Nat Ecol Evol 6, 763–773 (2022). https://doi.org/10.1038/s41559-022-01730-1

Papers to be published soon:

Dorottya Kalapis, Károly Kovács, Balázs Papp: The constructive role of compensatory evolution in phenotypic divergence
https://drive.google.com/file/d/1UD2JNQZBiILvJhMLiK5ZeTLKl4EWmiu2/view?usp=sharing

Roland Tengölics*, Balázs Szappanos*, Gábor Grézal, Dorottya Kalapis, Dóra Spekhárdt, Balázs Bálint, Laszló G. Nagy, Michael Mülleder, Enrica Calvani, Markus Ralser, Balázs Papp: Tempo and genomic basis of metabolome evolution in yeasts

Most important conference lectures and posters presented during the project period

Unraveling the impact of compensatory evolution on metabolic divergence
20th International Conference on Systems Biology in Okinawa, Japan 2019 – oral presentation

Unraveling the impact of compensatory evolution on metabolic divergence
Dorottya Kalapis, Roland Tengölics, Balazs Szappanos, Stefania Erdei, Balazs Papp -Darwin Days 2020 International Conference, Tihany, Hungary 2020 - poster

Unraveling the impact of compensatory evolution on metabolic divergence
Dorottya Kalapis, Károly Kovács, Roland Tengölcs, Fanni Birtyik , Balázs Papp - Congress of the European Society for Evolutionary Biology (ESEB 2022) Prague, Czech Republic 2022 - poster

Citations

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