Ph.D. thesis summary

Investigating the role of phenotypic heterogeneity in adaptive evolution

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Szeged, Hungary

2017

Introduction

Phenotypic heterogeneity is a phenomenon that stochastically increases the set of nonheritable phenotypic variations in a population containing genetically identical cells. By generating extreme phenotypic traits, phenotypic heterogeneity is generally assumed to provide only a fast physiological response against various kinds of stress factors, mainly in fluctuating environments. However, some scholars have also argued that stochastically generated variation precedes genetic changes and thereby facilitate the evolution of complex traits. This idea has remained disputed, not least because of the shortage of experimental studies.

Aims

During my PhD work, our main goal was to demonstrate that phenotypic heterogeneity not only influences survival in fluctuating environments, but also has a long-lasting impact on adaptive evolution. In order to reach

our goals, we integrated synthetic biology, experimental evolution and genomic analysis. We studied phenotypic heterogeneity that arises from stochastic fluctuation in cellular states and focused on the impact of such nongenetic cellular variation under permanent challenges in a novel stressful environment.

Methods:

Classical microbiological methods:

- Maintenance of yeast strains
- Laboratory evolution (to measure extinction rate and adaptation rate) by serial passaging of yeast populations

Molecular methods:

- Cloning
- Isolation of total DNA from yeast cell
- Isolation of plasmid DNA from bacterial cells
- Electroporation of bacteria
- Transformation of yeast (allelic replacement method)

- Promoter-swapping by double-joint PCR
- Modifications of yeast strains by CRISPR-Cas9-mediated genome engineering
- Cappilary and Whole Genome Sequencing (Illumina platform)

Methods for characterization of yeast strains:

- flow cytometry (to measure the fluorescence distribution of Pdr5p-GFP across cells from yeast cell populations)
- determination of population size
- determination of mutation rate (standard fluctuation assay to measure spontaneous canavanine resistance)
- MIC (minimum inhibitory concentration) determination by standard linear broth dilution technique in 96-well microtiter plate
- high-throughput fitness measurement by monitoring the optical density of yeast liquid cultures at 600 nm using 384-well density microtiter plates

Results

1. Generating a model system to investigate phenotypic heterogeneity

We developed two versions of an inducible synthetic gene circuit that generate varying degrees of expression stochasticity of an antifungal resistance gene, the plasma membrane efflux pump PDR5. In the strain without a positive feedback loop (noPF), most cells produced PDR5 with values very close to the average, displaying a unimodal distribution of low variability. In the strain carrying a positive feedback loop (PF), the variability was high and presented a bimodal distribution of PDR5 production – some cells produced very little PDR5, others produced a lot. This experimental setup allowed us to investigate directly the mechanisms through which heterogeneity can increase the survival of a population in harmful environment.

2. Phenotypic heterogeneity affects the outcome of laboratory adaptation

Next, we investigated how these two genetic circuits influence evolutionary adaptation towards antifungal stress and what the underlying molecular mechanisms of adaptation might be. Specifically, we asked how the level of nongenetic cellular variation shapes mutational effects. Two complementary experiments were used to study the impact of phenotypic heterogeneity on adaptive evolution. Experiment A measured extinction rate of the evolving strains as a function of gradually increasing fluconazole dosage, while experiment B aimed to maximize the fluconazole resistance increment during a fixed time period. Phenotypic heterogeneity promoted evolvability, as populations with high phenotypic heterogeneity reached a higher level of resistance and were less likely to become extinct during the course of laboratory evolution experiments. Next, we investigated the underlying molecular mechanisms of adaptation.

3. Phenotypic heterogeneity does not affect mutational supply

Based on a prior theoretical work, we hypothesized that phenotypic heterogeneity has a long-lasting impact on adaptive evolution for two possible reasons. Phenotypic heterogeneity may increase population size and hence the chance of occurrence of adaptive mutations. Alternatively, by generating individuals with exceptionally high trait values, phenotypic heterogeneity may increase the net adaptive value of beneficial mutations at an early stage of adaptation.

First, we tested the impact of phenotypic heterogeneity on the mutational supply that is jointly determined by the beneficial mutation rate and effective population size. We found no evidence that the more efficient adaptation would reflect higher mutational supply associated with phenotypic heterogeneity, as neither the population size nor the mutation rate (either local or global mutation rate) was different between the two strains.

4. Phenotypic heterogeneity shapes mutational effects

We asked how elevated mutational supply affects the outcome of laboratory evolution. We manipulated genomic mutation rate by inactivating a mismatch-repair gene (MSH2) both in the original noPF and PF strains, leading to an approximately 10-fold increase in genomic mutation rate. We initiated laboratory evolution with the mutator and nonmutator strains, as described previously (Experiment B). As expected, mutator strains (Δmsh2) reached higher levels of fluconazole resistance than the corresponding nonmutators that carried the same genetic circuit. More surprisingly, the level of resistance in the evolved noPF mutator strains was consistently lower than that in the evolved PF nonmutator strains. This suggests that despite massive increase in mutational supply, the genotype with low phenotypic heterogeneity has an intrinsic disadvantage during evolutionary adaptation. We concluded that the observed low adaptation rate under low phenotypic heterogeneity cannot be explained by shortage of mutational supply only. Phenotypic heterogeneity may enlarge the phenotypic effects of

mutations and consequently increase the set of adaptive mutations that provide resistance above a critical fluconazole dosage.

We tested whether the phenotypic effects of the mutations that accumulated during the course of laboratory evolution were contingent on phenotypic heterogeneity. First, we focused on the multidrug transporter PDR5, not least because this gene was mutated in all of the sequenced strains. A randomly selected nonsynonymous mutation (His595Asp) — observed in one of the evolved PF strains — was inserted individually both into the ancestor PF and noPF strains. The mutation conferred a highly significant decline in fluconazole susceptibility when phenotypic heterogeneity was high, but its beneficial effect was substantially reduced otherwise.

Next, we eliminated the positive feedback loop in the evolved PF strains, and as a result, tested the resistance level of evolved strains displaying a Pdr5p distribution reminiscent of the noPF strains (reduced expression stochasticity with similar mean expression). Elimination of heterogeneity substantially reduced the

resistance level acquired during the course of laboratory evolution. This result suggests that phenotypic heterogeneity may enlarge the set of adaptive mutations that provide resistance above a critical stress level. Additionally we observed that high phenotypic heterogeneity was beneficial over low heterogeneity, higher mean expression level setting in certain evolved strains. The same experimental setting increased resistance in the ancestor strain, as expected.

5. Phenotypic heterogeneity alleviates the fitness cost of acquired resistance

What might be the long-term advantage of phenotypic heterogeneity over mutations that simply provide a shift towards higher mean expression level? High expression of a drug-resistance gene provides resistance, but it also induces an especially high fitness cost in nonstressed conditions. We hypothesized that the ultimate fate of elevated phenotypic heterogeneity should reflect a fundamental trade-off between the level of resistance and the fitness cost of resistance: compared to constitutively

high expression level, phenotypic heterogeneity may dampen fitness costs when the level of fluconazole stress is relatively mild. To investigate this issue experimentally, we tested how the modulation of PDR5-GFP mean expression level and simultaneous removal of gene expression stochasticity affect fitness under stress conditions. Careful adjustment of the inducer level allowed us to generate expression settings with low phenotypic heterogeneity but exceptionally high or low mean PDR5-GFP expression levels. Under a wide-range of fluconazole dosages, fitness in the high phenotypic heterogeneity setting was higher than in the low phenotypic heterogeneity settings (high or low mean PDR5-GFP expression levels). We suspect that this reflects an intricate balance between the level of resistance conferred and the fitness cost of resistance-bearing mutations.

Summary

In sum, we demonstrated that cell-to-cell phenotypic heterogeneity could initiate key steps of microbial drug

resistance, for example by promoting fluctuations of protein concentrations in efflux pumps. Phenotypic heterogeneity promoted evolvability, partly by modulating the adaptive value of beneficial mutations. Therefore, we found experimental evidences that stochastically generated variation precedes genetic changes and thereby facilitate the evolution of complex traits. We also note that phenotypic heterogeneity can readily change in the laboratory. Therefore, different forms of phenotypic heterogeneity in nature may evolve as a direct response to novel and extreme environmental challenges.

List of publications

MTMT number: 10052465

1.1. Publication related to the Ph D. thesis

Bódi Z, Farkas Z, Nevozhay D, Kalapis D, Lázár V, Csörgő B, Nyerges A, Szamecz B, Fekete G, Papp B, Araujo H, Oliveira JL, Moura G, Santos MAS, Székely T, Balázsi G, Pál Cs. **Phenotypic heterogeneity promotes adaptive evolution** PLOS BIOLOGY 15:(5) Paper e2000644. 26 p. (2017)
IF: 9.797

1.2. Other publications

1. Diao JC, Charlebois DA, Neyozhay D, Bódi Z, Pál Cs, Balazsi G. **Efflux Pump Control Alters Synthetic Gene Circuit Function** ACS SYNTHETIC BIOLOGY 5:(7) pp. 619-631. (2016)
   IF: 5.382

2. Kalapis D, Bezerra AR, Farkas Z, Horváth P, Bódi Z, Daraba A, Szamecz B, Gut I, Bayes M, Santos MAS, Pál Cs. **Evolution of Robustness to Protein Mistranslation by Accelerated Protein Turnover** PLOS BIOLOGY 13:(11) Paper e1002291. 28 p. (2015)
   IF: 8.66