REPORT DOCUMENTATION PAGE

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14. ABSTRACT
We know much about the consequences of adaptation, but little of the underlying molecular causes. Our program combined biochemical, biophysical and evolutionary experiments to rigorously examine the molecular and biochemical basis of adaptation, mediated by changes in pyruvate kinase found in Richard Lenski's E. coli long-term evolution experiment. We have demonstrated, for the first time, that all the pykF mutations found in the E. coli long-term evolution experiment confer a significant adaptive fitness advantage to the bacterium. Moreover, we

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Report Title

Final Report: Research Area 14.3 Microbiology and Biodegradation. Attn: Wally Buchholz Title: Not different, Just Better: the adaptive evolution of an enzyme

ABSTRACT

We know much about the consequences of adaptation, but little of the underlying molecular causes. Our program combined biochemical, biophysical and evolutionary experiments to rigorously examine the molecular and biochemical basis of adaptation, mediated by changes in pyruvate kinase found in Richard Lenski's E. coli long-term evolution experiment. We have demonstrated, for the first time, that all the pykF mutations found in the E. coli long-term evolution experiment confer a significant adaptive fitness advantage to the bacterium. Moreover, we have shown that the mutations are likely to have a similar proximal basis of the benefit they confer: a shorter lag phase of the growth cycle. Not only are adaptive mutations often parallel at the physiological and genetic level, they are also parallel at the molecular level. The key finding from our program is that while the adaptive mutations in pyruvate kinase significantly alter the catalytic and allosteric function of the enzyme, they do not change the overall structure of the proteins or their thermal stability. Instead, we have demonstrated (using cutting edge deuterium exchange studies and molecular dynamic simulations) that the dynamics of key loops surrounding the active site are altered, which explain the changes in catalytic function.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Tim F. Cooper (2015) Generalists, specialists and enzyme evolution. Beijing Normal University workshop on microbial ecology.

Renwick Dobson (2014) Not different, just better: the adaptive evolution of a glycolytic enzyme. Canberra, Australia: COMBIO2014, 28 Sept - 2 Oct 2014.

Katherine Donovan (2014) Microfluidics enabled time-resolved electrospray ionization mass spectrometry coupled to hydrogen deuterium exchange (TR-ESI MS/HDX). MWC Future Science Day, Maurice Wilkins Centre, University of Auckland, 24 Nov 2014.

Katherine Donovan (2014) Enzyme dynamics in an adaptive enzyme. BIC Symposium, Biomolecular Computation, University of Canterbury, 28 Oct 2014.

Katherine Donovan (2014) Not different, just better: the adaptive evolution of a key glycolytic enzyme. Annual Biology Conference, School of Biological Sciences, University of Canterbury, 24 Oct 2014.

Katherine Donovan (2014) Structural, functional and dynamic analysis of an evolved enzyme. Trent Conference on Mass Spectrometry, Orillia, Ontario, Canada, 11-14 Aug 2014

Katherine Donovan (2014) Not different, just better: the adaptive evolution of a key glycolytic enzyme. Protein Engineering Canada Conference, Ottawa, Ontario, Canada, 20-22 June 2014.

Tim F. Cooper (2014) Anatomy of a beneficial mutation. Japanese society of evolutionary biology, Osaka, Japan.

Renwick Dobson (2013) Not different, just better: adaptations in a glycolytic enzyme. Christchurch, NZ: BIC Symposium, 23 Aug 2013.

Renwick Dobson (2013) Not different, just better: the adaptive evolution of a glycolytic enzyme. Denver, USA: Mechanisms of Protein Evolution, 7-9 Feb 2013.

Renwick Dobson (2013) Not different, just better: the adaptive evolution of a glycolytic enzyme. Australian Society for Biophysics Annual Meeting, Melbourne, Australia, 24-27 Nov 2013.

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Sarah Kessans (2014) The adaptive evolution of a glycolytic enzyme. Wellington, New Zealand: NZMS/NZSBMB Joint Meeting, 19 November 2014. (Conference Contributions - Oral presentations)

Sarah Kessans (2014) Not different, just better: the evolution of pyruvate kinase. Christchurch, New Zealand: BIC Connections and Collaborations Symposium, 17 June 2014. (Conference Contributions - Oral presentations)

Sarah Kessans (2013) Not different, just better: the adaptive evolution of a glycolytic enzyme. Queenstown, New Zealand: Queenstown Molecular Biology Conference, Enzyme Engineering and Evolution, 26 August 2013. (Conference Contributions - Oral presentations)

Number of Presentations: 16.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received		Paper
12/19/2015	1.00	Sarah C. Atkinson, Katherine A. Donovan, Sarah A. Kessans, Fen Peng, Tim F. Cooper, Michael D.W. Griffin, Geoffrey B. Jameson, Renwick C.J. Dobson. Grappling with anisotropic data, pseudo-merohedral twinning and pseudo- translational non-crystallographic symmetry: A case study involving pyruvate kinase., Acta Crystallographica Section D Biological Crystallography (04 2015)
12/19/2015	2.00	Katherine A. Donovan, Shaolong Zhu, Peter Liuni, Fen Peng, Sarah A. Kessans, Derek J. Wilson, Renwick C.J. Dobson. Conformational Dynamics and Allostery in Pyruvate Kinase, Journal of Biological Chemistry (05 2015)

TOTAL: **2**

Number of Manuscripts:

Books

Received Book

TOTAL:

Received Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

i. Katherine Donovan's PhD Thesis (Dancing to a different tune: adaptive evolution fine-tunes protein dynamics) was recommended for Distinction by the external reviewer (Assoc. Prof. David Ackerley).

ii. Best Poster award, Katherine Donovan, Conformational dynamics and allostery in pyruvate kinase activation. 40th Lorne Protein Conference, Lorne, Victoria, Australia. (Judged by Prof. Birte Höcker, Max Planck Institute for Development Biology)

iii. Best Poster award, Katherine Donovan, The adaptive evolution of a key glycolytic enzyme. Queenstown Molecular Biology Conference, Queenstown, New Zealand. (Judged by Prof. Shelley Copley, Colorado State University, USA)

Graduate Students

NAME	PERCENT_SUPPORTED	Discipline
Katherine Donovan	1.00	
Fen Peng	1.00	
Rosy Shaw	0.20	
Elena Surgrue	0.20	
Kristina Duan	0.50	
FTE Equivalent:	**2.90**	
Total Number:	**5**	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Sarah A Kessans	1.00
FTE Equivalent:	**1.00**
Total Number:	**1**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Renwick CJ Dobson	0.05	
Tim F Cooper	0.05	
FTE Equivalent:	**0.10**	
Total Number:	**2**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Anji Stampfli	1.00	Biosciences
FTE Equivalent:	**1.00**	
Total Number:	**1**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 1.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:...... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:...... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):...... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:...... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields:...... 0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME
Katherine A. Donovan
Fen Peng

Total Number: **2**

Names of other research staff

NAME	PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See Attachment

Technology Transfer

FINAL REPORT: Contract W911NF- 11-1-0481, “Not different, Just Better: the adaptive evolution of an enzyme”

1) Summary

We know much about the consequences of adaptation, but little of the underlying molecular causes. Our program combined biochemical, biophysical and evolutionary experiments to rigorously examine the molecular and biochemical basis of adaptation, mediated by changes in pyruvate kinase found in Richard Lenski’s *E. coli* long-term evolution experiment. This program was aimed at uncovering the molecular basis for a series of adaptive mutations in a key allosteric enzyme. We chose the enzyme pyruvate kinase (gene *pykF*) from *Escherichia coli* as our model system, since 1) it catalyzes a reaction central to energy metabolism, 2) it is precisely regulated by allostery and the adaptation of allostery is unknown, and 3) multiple experiments by others have demonstrated that adaptive mutations reproducibly occur in this enzyme when *E. coli* is grown under low glucose conditions.

We have demonstrated, for the first time, that all the *pykF* mutations found in the *E. coli* long-term evolution experiment confer a significant adaptive fitness advantage to the bacterium. Moreover, we have shown that the mutations are likely to have a similar proximal basis of the benefit they confer: a shorter lag phase of the growth cycle. Not only are adaptive mutations often parallel at the physiological and genetic level, they are also parallel at the molecular level. By measuring the effect of *pykF* mutations in a range of resources that have different characterized uptake mechanisms, we have made strides in determining the underlying molecular basis of their benefit.

The key finding from our program is that while the adaptive mutations in pyruvate kinase significantly alter the catalytic and allosteric function of the enzyme, they do not change the overall structure of the proteins or their thermal stability. Instead, we have demonstrated (using cutting edge deuterium exchange studies and molecular dynamic simulations) that the dynamics of key loops surrounding the active site are altered, which explain the changes in catalytic function. *Thus, we propose that protein dynamics is an important and general underlying molecular cause of adaptation in enzymes.*

Serendipitously, we solved the structure of *E. coli* pyruvate kinase A301S with and without its allosteric effector (fructose-1,6-bisphosphate), allowing us to propose a mechanism of allosteric regulation in this enzyme. In particular, the binding of fructose-1,6-bisphosphate causes a change in the relative orientation of domains within the monomer, which in turn affects the dynamics of key active site loops involved in substrate binding. Deuterium exchange studies support our hypothesized mechanism, and also demonstrate that the allosteric signal is communicated from the allosteric binding site to the active site by destabilization of the beta-strands that form the barrel in the $(\alpha/\beta)_8$-barrel active site domain.

2) We have achieved each of our stated goals

Question 1: How do the effects of adaptive mutations depend on their genetic background?

- ***Aim 1)*** *Allelic replacement of the evolved and ancestral alleles in the ancestral and evolved genetic backgrounds.* **Achieved**
- ***Aim 2)*** *Quantifying and comparing the fitness of the various* pykF *alleles in different genetic backgrounds.* **Achieved**

Question 2: What is the repeatability of molecular evolution? That is, are adaptive mutations in the same gene, but replicate populations, functionally parallel?

- ***Aim 3)*** *Expression, purification and functional analysis of evolved pyruvate kinase type 1 enzymes.* **Achieved**
- ***Aim 4)*** *Investigating the structural changes in the evolved pyruvate kinase type 1 enzymes.* **Achieved**
- ***Aim 5)*** *Unraveling the metabolic consequences of the evolved pyruvate kinase type 1 enzymes.* **Achieved**

Question 3: Do the adaptive pyruvate kinase mutations have secondary effects?

- ***Aim 6)*** *Determine the fitness of evolved pyk*F *alleles in novel resources.* **Achieved**

Thesis and Manuscript preparation – Driven by PI Dobson and Cooper. **On Track**

The ARO contract has supported the following staff and students:

- 1 Postdoctoral Fellow (Dr Sarah Kessans, University of Canterbury)
- 2 PhD students (now Dr Fen Peng, University of Houston; Dr Katherine Donovan, UC)
- 3 Honours students (Elena Sugrue, UC; Rosie Shaw, UC; Kristina Duan, UoH)
- 1 Summer studentship (Anji Stampfli, UC).

3) Outputs

A) Publications in journals:

1) Katherine A. Donovan, Sarah C. Atkinson, Sarah A. Kessans, Fen Peng, Tim F. Cooper, Michael D.W. Griffin, Geoffrey B. Jameson and Renwick C.J. Dobson (2015) **Grappling with anisotropic data, pseudo-merohedral twinning and pseudo-translational non-crystallographic symmetry: A case study involving pyruvate kinase.** Provisionally accepted *Acta Crystallographica D: Biological Crystallography*
2) Katherine A. Donovan, Shaolong Zhu, Peter Liuni, Fen Peng, Sarah Kessans, Tim F. Cooper, Derek J. Wilson, Renwick C.J. Dobson (2015) **Conformational dynamics and allostery in pyruvate kinase.** Provisionally accepted *Journal of Biological Chemistry.*
3) Katherine A. Donovan, Sarah C. Atkinson, Sarah A. Kessans, Fen Peng, Tim F. Cooper, Michael D.W. Griffin, Geoffrey B. Jameson and Renwick C.J. Dobson (2015) **A structural explanation for allostery in pyruvate kinase.** Drafted and will be submitted to *Acta Crystallographica D: Biological Crystallography*
4) Katherine A. Donovan, Fen Peng, Sarah Kessans, Tong Zhu, Shaolong Zhu, Ben Porebski, Silas Villas-Boas, Ashley M. Buckle, Derek J. Wilson, Tim F. Cooper, Renwick C.J. Dobson (2015) **Dancing to different tune: Natural selection of an enzyme by adaptation fine-tunes protein dynamics.** To be drafted and will be submitted to *Science.*
5) Elena Sugrue, Katherine A. Donovan, Sarah Kessans, Tim F. Cooper, Renwick C.J. Dobson (2015) **The dynamic lid domain of pyruvate kinase: not involved in allostery, but critical for reaction promiscuity.** To be drafted based on Elena Sugrue’s Honours report and submitted to FEBS Letters.
6) Fen Peng, Renwick C.J. Dobson and Tim F. Cooper (2016) **The benefit of parallel beneficial mutations in *pykF* depends strongly on their evolved genetic backgrounds.** In preparation, to be submitted to *Evolution.*
7) Fen Peng, Kristina Duan, Katherine A. Donovan, Sarah Kessans, Renwick C.J. Dobson and Tim F. Cooper (2016) **Independently selected mutations in *pykF* confer a benefit by facilitating rapid initiation of glucose uptake.** In preparation, to be submitted to *Molecular Biology and Evolution.*
8) Rosie Shaw, Katherine Donovan, Regan Clark, Fen Peng, Tim F. Cooper, Anthony Poole, Renwick C.J. Dobson (2016) **Adaptive evolution by altering quaternary structure.** To be drafted based on Rosie Shaw’s Honours report and submitted to FEBS Letters.

B) Academic theses and honours reports:

9) Fen Peng (2015) **Molecular basis of adaptive evolution of pyruvate kinase in *Escherichia coli*.** PhD Thesis, University of Houston (Houston, USA).
10) Katherine Donovan (2015) **Dancing to a different tune: adaptive evolution fine-tunes protein dynamics.** PhD Thesis, University of Canterbury (Christchurch, New Zealand).
11) Kristina Duan (2016) **Evolved PykF enzymes confer an adaptive benefit by promoting rapid initiation of glucose uptake.** Ongoing Honours Report to be submitted in 2016, University of Houston (Houston, USA).
12) Rosie Shaw (2014) **Pyruvate kinase as an adaptive focus in the absence of translation initiation signals.** Honours Report, University of Canterbury (Christchurch, New Zealand).
13) Elena Sugrue (2012) **Investigation into the role of a dynamic domain in pyruvate kinase.** Honours Report, University of Canterbury (Christchurch, New Zealand).

C) Invited seminars:

Ci) Conferences and Symposia:

14) Tim F. Cooper (2015) **Generalists, specialists and enzyme evolution**. Beijing Normal University workshop on microbial ecology.

15) Renwick Dobson (2014) **Not different, just better: the adaptive evolution of a glycolytic enzyme.** Canberra, Australia: COMBIO2014, 28 Sept - 2 Oct 2014.

16) Katherine Donovan (2014) **Microfluidics enabled time-resolved electrospray ionization mass spectrometry coupled to hydrogen deuterium exchange (TR-ESI MS/HDX).** MWC Future Science Day, Maurice Wilkins Centre, University of Auckland, 24 Nov 2014.

17) Katherine Donovan (2014) **Enzyme dynamics in an adaptive enzyme.** BIC Symposium, Biomolecular Computation, University of Canterbury, 28 Oct 2014.

18) Katherine Donovan (2014) **Not different, just better: the adaptive evolution of a key glycolytic enzyme.** Annual Biology Conference, School of Biological Sciences, University of Canterbury, 24 Oct 2014.

19) Katherine Donovan (2014) **Structural, functional and dynamic analysis of an evolved enzyme.** Trent Conference on Mass Spectrometry, Orillia, Ontario, Canada, 11-14 Aug 2014

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24) Renwick Dobson (2013) **Not different, just better: the adaptive evolution of a glycolytic enzyme.** Australian Society for Biophysics Annual Meeting, Melbourne, Australia, 24-27 Nov 2013.

25) Katherine Donovan (2013) **The adaptive evolution of a key glycolytic enzyme.** Annual Biology Conference, School of Biological Sciences, University of Canterbury, 17 Oct 2013.

26) Sarah Kessans (2015) **The dynamics of change: the adaptive evolution of an allosterically-regulated enzyme in *E. coli*.** Christchurch, New Zealand: BIC Evolving Symposium, 15 June 2015. (Conference Contributions - Oral presentations)

27) Sarah Kessans (2014) **The adaptive evolution of a glycolytic enzyme.** Wellington, New Zealand: NZMS/NZSBMB Joint Meeting, 19 November 2014. (Conference Contributions - Oral presentations)

28) Sarah Kessans (2014) Not different, just better: the evolution of pyruvate kinase. Christchurch, New Zealand: BIC Connections and Collaborations Symposium, 17 June 2014. (Conference Contributions - Oral presentations)

29) Sarah Kessans (2013) Not different, just better: the adaptive evolution of a glycolytic enzyme. Queenstown, New Zealand: Queenstown Molecular Biology Conference, Enzyme Engineering and Evolution, 26 August 2013. (Conference Contributions - Oral presentations)

Cii) Institutions

30) Katherine Donovan (2015) **Dancing to a different tune: adaptive evolution fine-tunes protein dynamics** Cancer Biology department at Dana-Farber Cancer Institute/Harvard Medical School, Massachusetts, USA, 24 Nov 2015.

31) Renwick Dobson (2015) **Not different, just better: The adaptive evolution of a glycolytic enzyme.** University of Auckland, 15 June 2015.

32) Renwick Dobson, R.C.J. (2015) **Not different, just better: The adaptive evolution of a glycolytic enzyme.** University of Houston, Houston, USA, 3 June 2015

33) Dobson, R.C.J. (2015) **Not different, just better: The adaptive evolution of a glycolytic enzyme.** University of York, Toronto, Canada, 8 June 2015.

34) Dobson R.C.J. (2013) **Enzymes: dynamics, quaternary structure and evolution**. Massey University, Institute of Molecular Biosciences, 18 Oct 2013.

35) Tim F. Cooper (2013) **Epistasis and the evolvability of microbial populations.** Rice University, Center for Theoretical Biological Physics department seminar.

36) Dobson R.C.J. (2013) **Enzymes: dynamics, quaternary structure and evolution**. University of Auckland, Medical School, 31 October 2013.

D) Poster presentations: (presenting author underlined)

37) Sarah Kessans, Katherine Donovan, Fen Peng, Tong Zhu, Sillas Villas-Boas, Derek Wilson, Tim Cooper, Renwick Dobson (2015) **The dynamics of change: the adaptive evolution of an allosterically-regulated enzyme in *E. coli*.** Queenstown Molecular Biology Conference, Proteins Satellite Meeting, 3 Sept 2015.

38) Katherine Donovan, Shaolong Zhu, Peter Liuni, Sarah A. Kessans, Derek J. Wilson, Renwick C.J. Dobson (2015) **Conformational dynamics and allostery in pyruvate kinase activation.** Queenstown Molecular Biology Conference, Queenstown, New Zealand.

39) Katherine Donovan, Shaolong Zhu, Peter Liuni, Sarah A. Kessans, Derek J. Wilson, Renwick C.J. Dobson (2015) **Conformational dynamics and allostery in pyruvate kinase activation.** 40th Lorne Protein Conference, Lorne, Victoria, Australia. **Poster prize

40) Katherine Donovan, Fen Peng, Sarah Kessans, Tim Cooper & Renwick Dobson (2014) **The adaptive evolution of a key glycolytic enzyme.** Lorne, Australian: 39th Lorne Protein Structure and Function Conference, 6-9 Feb 2014.

41) Katherine Donovan, Fen Peng, Sarah Kessans, Tim Cooper & Renwick Dobson (2014) **Not different, just better: the adaptive evolution of a key glycolytic enzyme.** 39th Lorne Protein Conference, Lorne, Victoria, Australia.

42) Pen Peng, Tim Cooper (2013) **Relating Genotype to Phenotype in the Adaptive Evolution of Pyruvate Kinase.** Society for Evolutionary Biology General Meeting, Snowbird, Utah, USA.

43) Katherine Donovan, Fen Peng, Tong Zhu, Sarah Kessans, Tim Cooper & Renwick Dobson (2013) **The adaptive evolution of a key glycolytic enzyme.** Queenstown Molecular Biology Conference, Queenstown, New Zealand. **Poster prize

44) Katherine Donovan, Sarah Kessans, & Renwick Dobson (2013) **Regulatory switch of a key glycolytic enzyme.** 38th Lorne Protein Conference, Lorne, Victoria, Australia.

4) Awards

i. Katherine Donovan's PhD Thesis (**Dancing to a different tune: adaptive evolution fine-tunes protein dynamics**) was recommended for Distinction by the external reviewer (Assoc. Prof. David Ackerley).

ii. Best Poster award, Katherine Donovan, **Conformational dynamics and allostery in pyruvate kinase activation.** 40th Lorne Protein Conference, Lorne, Victoria, Australia. (Judged by Prof. Birte Höcker, Max Planck Institute for Development Biology)

iii. Best Poster award, Katherine Donovan, **The adaptive evolution of a key glycolytic enzyme.** Queenstown Molecular Biology Conference, Queenstown, New Zealand. (Judged by Prof. Shelley Copley, Colorado State University, USA)

Introduction

***How* do organisms adapt to novel environments?** Adaptation is the process by which a population moves towards a phenotype that represents a better fit to the environment. We know a great deal about the *consequences* of adaptation; for example, the speed with which adaptive mutations of certain fitness effects will spread in a population and how this will be affected by changing population parameters (size, recombination, etc). In contrast, we know very little about the underlying *molecular causes* of adaptation; that is, how mutations act to produce an organism’s phenotype. Indeed, with few exceptions, such as the emergence of drug resistance, the link between genetic changes and their effect on fitness has been left as a black box—mutations go in, the phenotype comes out. Answers to fundamental questions are not just unknown, but unexplored. Understanding the mapping between the molecular consequences of adaptive mutations and their phenotypic consequences will allow us to ask new types of questions: 1) How do the effects of an adaptive mutation depend on genetic background? 2) Are independent adaptive mutations in the same gene functionally parallel; that is, do they confer the same function to the evolved protein? 3) What role does protein stability play in constraining the ability of an enzyme to adapt?

Testing models of adaptation poses several challenges—not least the long time scales that can be required for adaptation to occur. To circumvent this limitation we build on a long-term laboratory study in which 12 independent replicate *E. coli* populations have been evolved in a glucose-limiting environment for 60,000 generations (~1 million human years!). During this time, the replicate populations increased in fitness relative to their common ancestor. Remarkably, the genetic changes underlying this adaptation were often parallel, being concentrated in relatively few genes. In particular, mutations in one gene, *pykF,* occurred independently (and were fixed) in all 12 long-term populations—a signature that they are likely to be adaptive. Moreover, in one case the same mutation in *pykF* occurred independently in three populations. *Our research program studied why* pykF *serves as an adaptive focus. To do this, we have developed a molecular ‘picture’ of the effect of the evolved changes on the structure and function of the gene product—pyruvate kinase.*

Pyruvate kinase catalyzes a key regulatory step in glycolysis—the biochemical pathway central to energy metabolism. The catalyzed reaction involves transfer of a phosphoryl group from phosphoenolpyruvate to ADP, generating the important biomolecules pyruvate and ATP. Tight regulation of glycolysis is crucial, since gluconeogenesis (the opposing process) would otherwise occur simultaneously, leading to wasteful cycling of biochemical intermediates. *We hypothesized that pyruvate kinase is a focal point for parallel evolution because it is a key control point in energy metabolism.* Although the adaptive mutations in PK1 are distributed throughout the gene sequence, when mapped onto the pyruvate kinase structure they clustered to three hot spots. *We predicted that the mutations would have substantial effects on the catalytic activity and/or allosteric regulation of the evolved enzymes, since the currently accepted mechanism of PK1 regulation occurs* via *rearrangement of the tetramer interfaces*.

Understanding and predicting the effect of mutations at the organismal level is a major challenge of evolutionary biology. Empirical data addressing why some mutations are adaptive (and, conversely, why most are not) will have major implications for our ability to predict and understand the outcome of evolution, both *in vitro* and *in vivo*.

Results to proposed questions and data.

***Question 1:* How do the effects of an adaptive mutation depend on genetic background?**

Summary:

We have discovered that the magnitude of the fitness benefit conferred by the adaptive mutations in pyruvate kinase depends on the background on which they arise, although they are almost always positive. This strongly suggests that pyruvate kinase is a generalist (in the sense that evolved mutations can confer benefits in many backgrounds), but is also involved in epistatic interactions. Moreover, we discovered that the different adaptive mutations are parallel in their effect, providing the first evidence that although the mutations are spread across the gene, their fitness effect is very likely to be the same.

For extended details **see Fen Peng’s PhD thesis, Chapter 2.**

- ***Aim 1)*** *Allelic replacement of the evolved alleles in the ancestral genetic background.* ***Achieved***

Detailed Results:

We constructed two series of strains that were designed to allow comparison of the effect of independently evolved mutations in *pyk*F in a common genetic background (the ancestor to our evolution experiment) and in the evolved background in which they are present in the evolution experiment. We found that the different point mutation alleles *of pyk*F had similar fitness effects in the ancestral background (Figure 1). Interestingly, these effects were significantly different from that of a deletion allele, clearly indicating that the mutant enzymes retain some function. The fitness of the evolved *pyk*F genes added to the ancestor increased in the range from 6% to 12% (see Figure 1).

Effects were generally higher and more variable in the evolved backgrounds, where they ranged from 0 to 25% (Figure 2). We draw one important conclusions from these results. The effects of focal mutations are not constant, but depend on the presence of additional mutations as they arise during evolution.

- ***Aim 2)*** *Quantifying and comparing the fitness of the various* pyk*F alleles in different genetic backgrounds.* **Achieved**

Detailed Results:

We tested two explanations for our finding that the evolved *pykF* enzymes had different fitness effects in the ancestor and in their evolved background (Figure 3): 1) they are caused by different pyruvate kinase enzymes, or 2) they are caused by the different genetic backgrounds in which they occur. To do this, we moved each of three *pyk*F alleles (WT, A301S, Del) into 11 different evolved backgrounds (Figure 4). We found that each of the transferred alleles had similar effects in the same background and very different effects across backgrounds.

Additional work:

The evolved alleles result in a shorter lag phase growth. As a first step in determining the mechanism underlying fitness effects of mutations in *pyk*F, we have analyzed growth curves of the series of strains made by adding the mutations in to the common ancestral background. We find a significant trend toward a decrease in lag time – the time taken for cells to begin growth following transfer to fresh medium (Figure 3A) – and a smaller and non-significant

trend toward faster growth rates (Figure 3B). These results are consistent with previous work proposing that *pyk*F mutations might provide a benefit to cells by allowing them to maintain higher PEP (phosphoenolpyruvate) – the substrate of the pyruvate kinase – levels during stationary phase, increasing the rate at which glucose can be imported into cells when it is added to the environment.

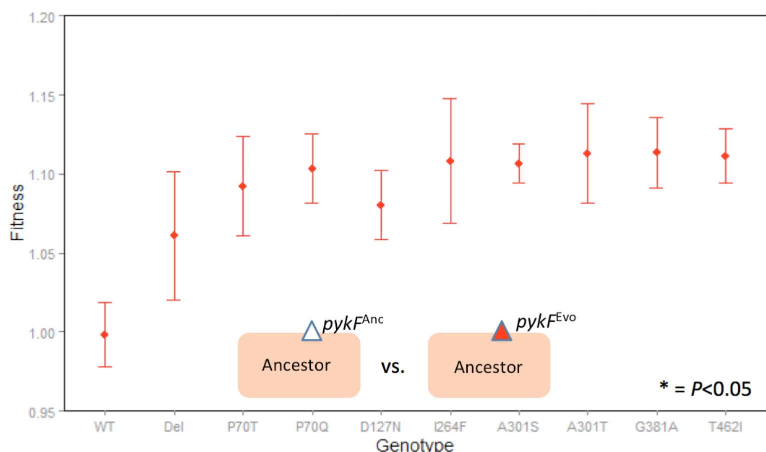

Figure 1: Fitness of ancestral and evolved *pyk*F genes in the ancestral genetic background. Symbols indicate mean and error bars indicate the 95% CI of three replicate fitness measurements.

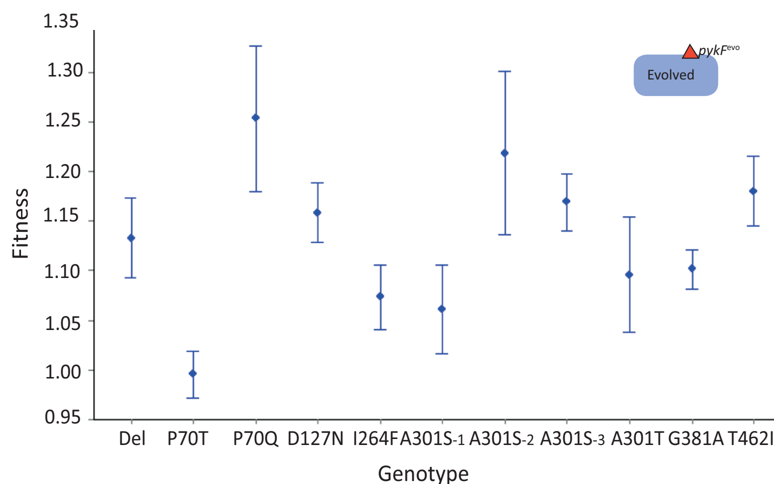

Figure 2: Fitness of evolved *pykF* genes in the evolved backgrounds. Symbols indicate mean and error bars indicate the 95% CI of three replicate fitness measurements.

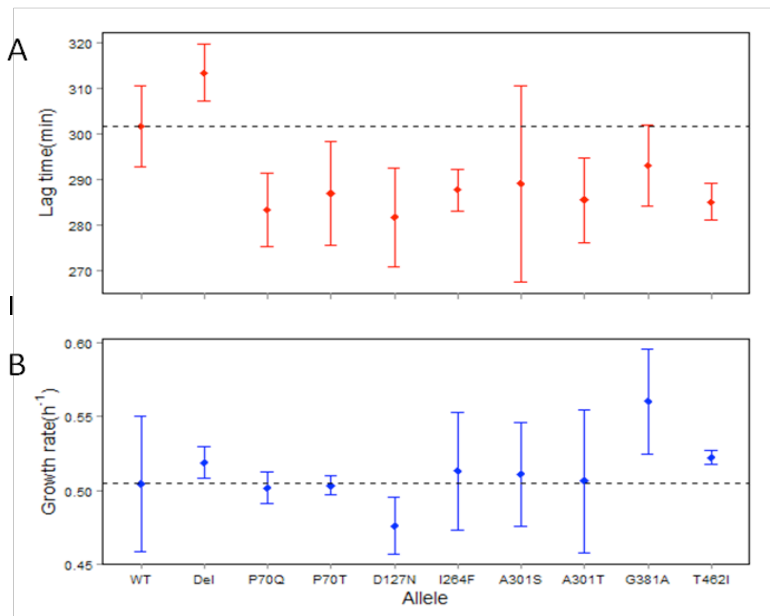

Figure 3: Lag time (A.) and growth rates (B.) of the evolved *pyk*F allele in the ancestor strain, compared directly with the ancestor. Symbols indicate mean and error bars indicate the 95% CI of three replicate fitness measurements. There is a significantly shorter time for evolved strains, except for the deletion.

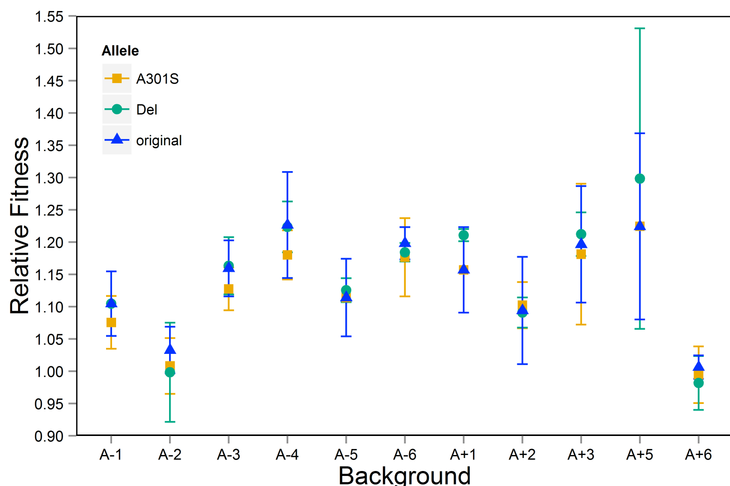

Figure 4. Fitness effects of different pyruvate kinase enzymes in 11 independently evolved genetic backgrounds. Different symbol colors indicate the effect of each of three *pyk*F genes in each genetic background. Genetic backgrounds are clones isolated from populations started from a common ancestor and evolved independently for 20,000 generations. Symbols indicate mean and error bars indicate the 95% CI of three replicate fitness measurements.

Question 2: Are adaptive mutations in the same gene, but replicate populations, functionally parallel?

Summary:

Following Question 1, in which we discovered that the adaptive mutations confer a parallel fitness advantage, we asked whether the evolved enzymes where themselves functionally altered in parallel (i.e. catalysis and regulation). We discovered that while the catalytic and allosteric function of the evolved enzymes was surprisingly different, the overall structure was surprisingly *un*altered. We then hypothesized that the dynamics of the enzymes would be different. We tested our hypothesis using hydrogen/deuterium exchange experiments, which demonstrates that the dynamics of the proteins are indeed different. Thus, we have demonstrated that, in this case, evolution alters enzyme dynamics. Lastly, consistent with the central role of pyruvate kinase in energy metabolism, we have shown that the metabolome is significantly altered by the adaptive mutations in pyruvate kinase. In particular, we have discovered that the TCA cycle and fatty acid biosynthesis are down regulated. We propose that the mutations optimize the metabolome for growth in a low glucose environment.

For extended details see **Katherine Donovan’s PhD thesis, Chapters 4, 5 and 6**.

- ***Aim 2)*** *Expression, purification and functional analysis of evolved pyruvate kinase type 1 enzymes.* ***Achieved***

Detailed Results:

Figure 5 demonstrates that the there are significant differences in the kinetic properties of the eight adaptive mutations when compared to the WT enzyme (i.e., the wild type ancestral enzyme). Importantly, the major effect is on PEP binding and interestingly, the enzymes show altered allostery with respect to both PEP and FBP. This is an exciting result and may represent the first observation of enzymes altering allostery to adapt to a new environment.

Historically, the view was that most mutations could be tolerated without loss of stability or function. A recent review highlighted a change in this view, with the rationalization that most mutations cause some change in stability, usually destabilizing. It has been proposed that some proteins contain inherent robustness (the threshold robustness model), such that adaptive mutations can be tolerated without compromising stability. Given that the adaptive mutations accumulated in relatively few genes in the LT experiment, and in particular in pyruvate kinase, the thermal stability of wild type pyruvate kinase was compared to the evolved enzymes (Figure 13). These studies show that the inherent thermo-stability of the pyruvate kinase enzyme is rather high at 62 ^{o}C and that the adaptive mutations do not have a significant affect on thermal stability. Our data are consistent with the threshold robustness model.

- ***Aim 3)*** *Investigation of structural changes of the evolved pyruvate kinase type 1 enzymes.* ***Achieved***

Detailed Results:

Given that several of the mutations (A301S, A301T and G381A) reside at the tetrameric interface, we investigated whether these adaptive mutations affected the oligomeric state of the enzymes. To examine the association/dissociation limit of wild type and the evolved pyruvate kinase enzymes, analytical ultracentrifugation experiments were performed (Figure 6). None of the mutant enzymes had altered quaternary function, even at low nM

concentrations. These data demonstrate that the adaptive mutations do not cause changes in the quaternary structure.

To examine the structural changes, we initiated atomic resolution crystallization studies on the mutant enzymes (resolution varying from 2–2.6 Å) comparing them to a wild-type structure that we determined in house. Surprisingly, each of the evolved enzymes overlay very closely with the wild type structure (Figure 7) and only very small changes could be found. This was surprising, given the very different kinetic and allosteric functions of the enzymes. We verified that in solution the mutation were not altering the structure of the protein using small angle X-ray scattering.

- ***Aim 4)*** *Unraveling the metabolic consequences of the evolved pyruvate kinase type 1 enzymes.* ***Achieved***

Finally, we collaborated with Dr Silas Villas-Boâs (University of Auckland) to explore how changes in pyruvate kinase affect the metabolome. The most significant changes in the metabolome are shown in Figure 8. Large increases in the concentrations of the extracellular TCA metabolites (succinate, malic acid, and fumaric acid) and fatty acids, suggest that flux through this pathway is low. Interestingly, although the changes are generally consistent across the mutations, there are significant differences with two mutations (P70T and P70Q, Figure 8B), which suggest that there is more than one way to optimize the metabolome. Overall, we interpreted the changes in the metabolome as a global optimization of metabolomic function to the low glucose environment. It is unsurprising that pyruvate kinase is an adaptive focus to this evolutionary pressure, given its central role in metabolism.

Additional work:

The adaptive mutations alter protein dynamics. Since the very different kinetics of the evolved pyruvate kinase enzymes (Figure 5), cannot be explained by the surprisingly similar structures when compared to the wild-type enzyme, we hypothesized that the changes in kinetics were caused by the mutations altering the dynamics of the proteins. In collaboration with A/Prof Derek Wilson (University of York, Canada), hydrogen/deuterium exchange mass spectrometry studies were undertaken to gauge whether the dynamics of the evolved enzymes was different to the wild-type enzyme. The results for these studies are consistent with our hypothesis: global deuterium uptake of the evolved enzymes is different to each other and to the wild type enzyme suggesting that they have different global dynamics (Figure 9). Using pepsin digests, we have shown that the pattern of deuterium exchange across the protein is also different (Figure 10). Supporting this finding are molecular dynamic simulation studies (Figure 11). This work is ongoing, but provides strong evidence that the evolved mutations are altering protein dynamics.

Unraveling the mechanism of allosteric activation of *E. coli* pyruvate kinase by FBP. Understanding how the mutations altered the allosteric mechanism, as seen in the kinetic studies (Figure 5), required the structure of pyruvate kinase with bound FBP. Soaking wild-type and A301T pyruvate kinase crystals with the allosteric effector caused immediate cracking of the crystals and no diffraction data could be collected. This phenomenon often occurs when the ligand binds to the protein, causing significant rearrangements within the protein, such that the protein interactions necessary to form the lattice of the crystal are broken. In contrast, pyruvate kinase A301S when soaked with FBP did not cause crystal cracking and diffraction could be collected to 2.6 Å. This structure (Figure 12) shows that upon binding of FBP, the orientation of the monomers within the tetramer is altered with respect to one another. Each twist by about 10 degrees and the interfaces of the tetramer are significantly changed. When we compared the active sites of the FBP bound structure with

the unbound structure, we notice very few differences, although two active site loops become very dynamic. This suggests to us that FBP binding remodels the active site to better accommodate PEP, and in so doing activates the enzyme. Importantly, this result is consistent with our finding that the dynamics of the active site loops are critical for function.

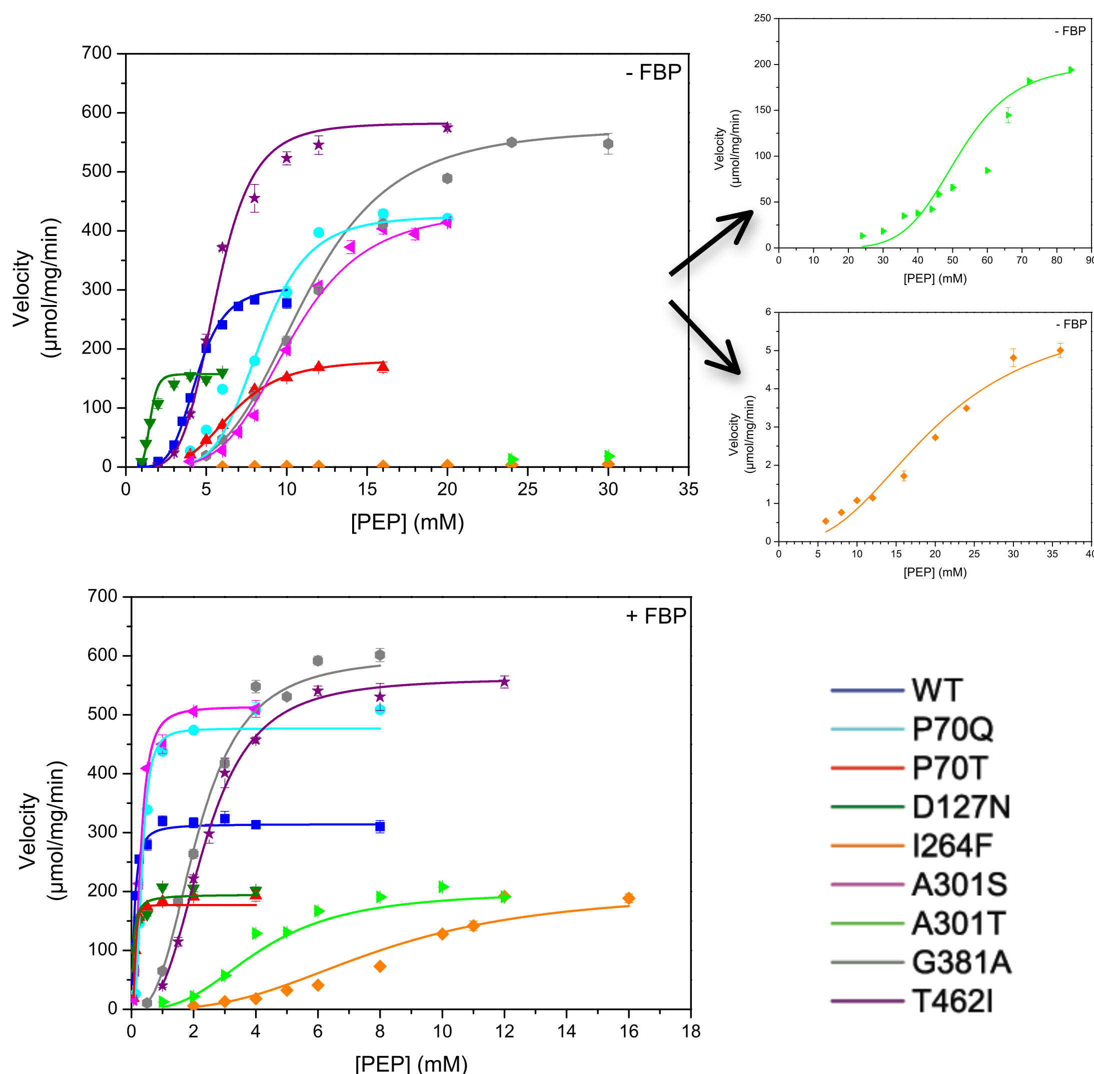

Figure 5: Enzyme kinetic plots of the wild type and mutant pyruvate kinase enzymes demonstrate that they are functionally very different from one another. These plots show the rate of reaction relative to increasing phosphoenol pyruvate (PEP) concentrations. The K_M, k_{cat}, and allosteric effect of FBP are different across this set of enzymes.

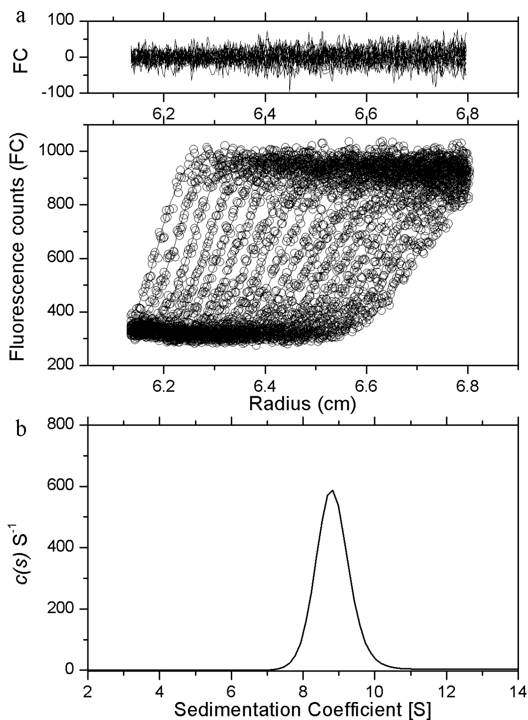

Figure 6: Analytical ultracentrifugation data demonstrates that the pyruvate kinase A301T is a tetramer even at low nM concentrations. The c(s) distribution (lower panel) shows only a single peak that is the same as the wild-type enzyme. The upper panels show the raw analytical ultracentrifugation data.

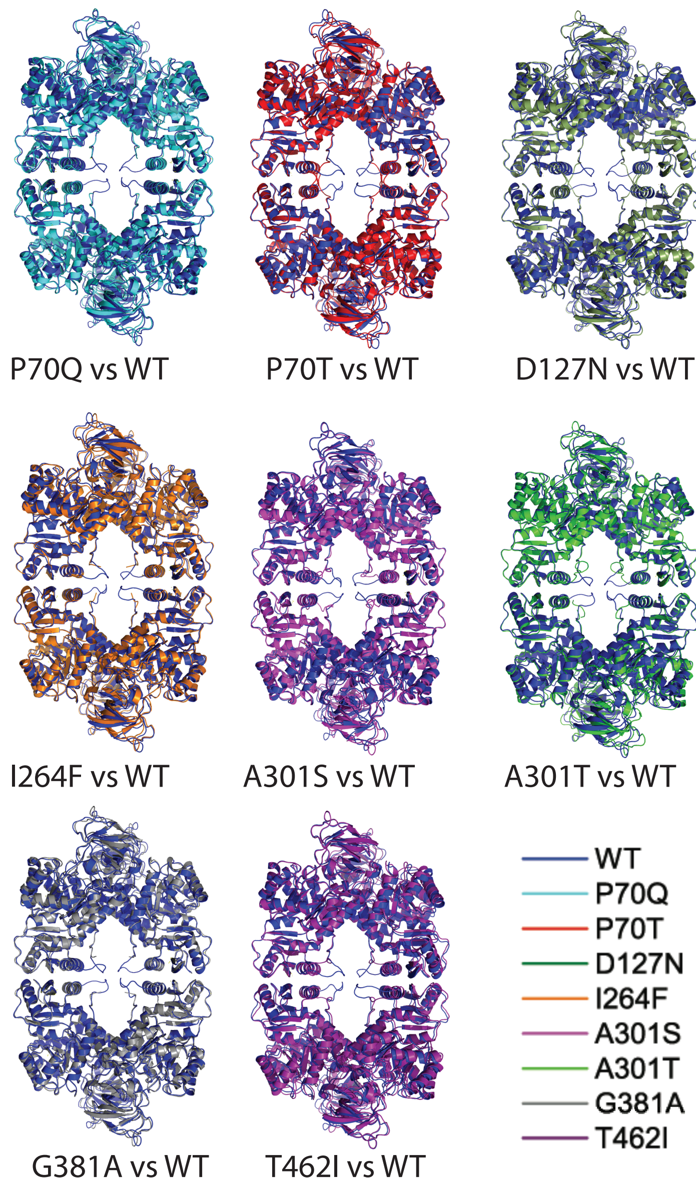

Figure 7: The crystal structures of each of the evolved pyruvate kinase enzymes compared to the wild-type enzyme reveal that the evolved enzymes are *very* similar in atomic structure. The resolution of the evolved pyruvate kinases ranged from 2.0 – 2.6 Å. The R_{free} statistics ranges from 18% - 25%. The adaptive mutation could be resolved in the electron density.

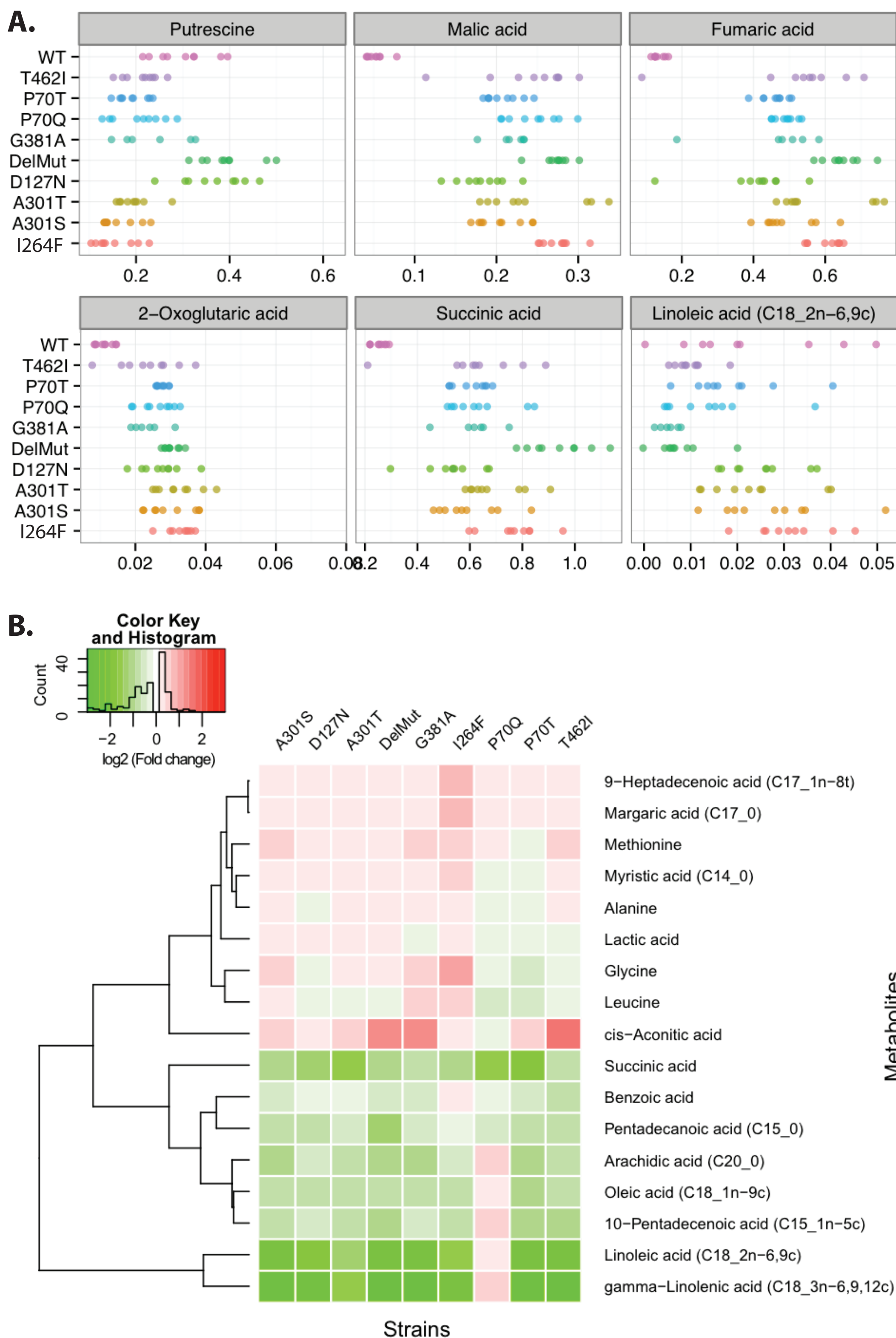

Figure 8. Pyruvate kinase mutations alter the cellular metabolome. A. Significant changes in the relative abundance of extracellular metabolites were focused on TCA cycle substrates (malic acid, fumaric acid, 2-oxoglutaric acid, and succinic acid; illustrating an increase in relative abundance in mutants compared to wild type cells), fatty acids (linoleic acid; varying across mutants and wild type), and putrescine (varying across mutants, but generally lower in abundance as compared to the wild type). B. Significant changes in the relative abundance of intracellular metabolites were clustered around fatty acids (generally lower abundances in mutants as compared to wild type), TCA cycle substrates (cis-aconitic acid higher in mutants, succinic acid lower in mutants), and amino acids (varied across mutants and wild type).

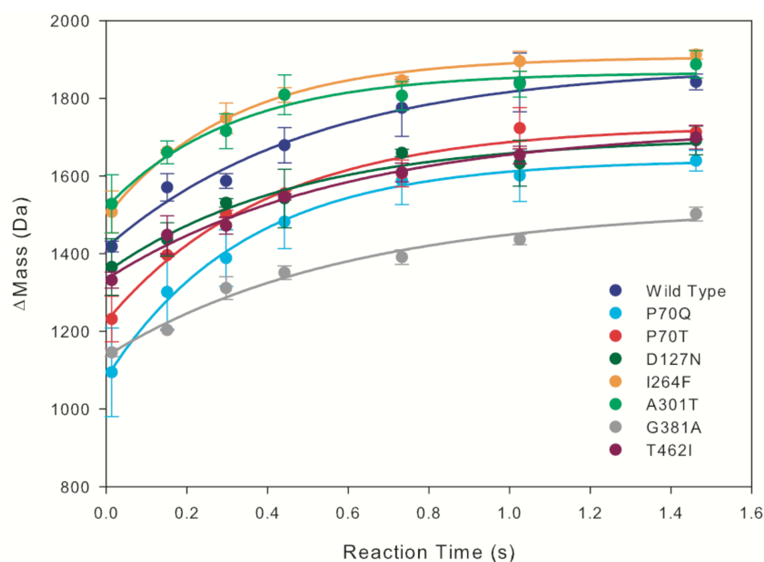

Figure 9. Global hydrogen/deuterium exchange studies for the pyruvate kinase enzymes demonstrate that the mutations cause a change in protein dynamics. This panel plots the uptake of deuterium versus time. The mutants show different initial uptake rates and different total uptake (plateau).

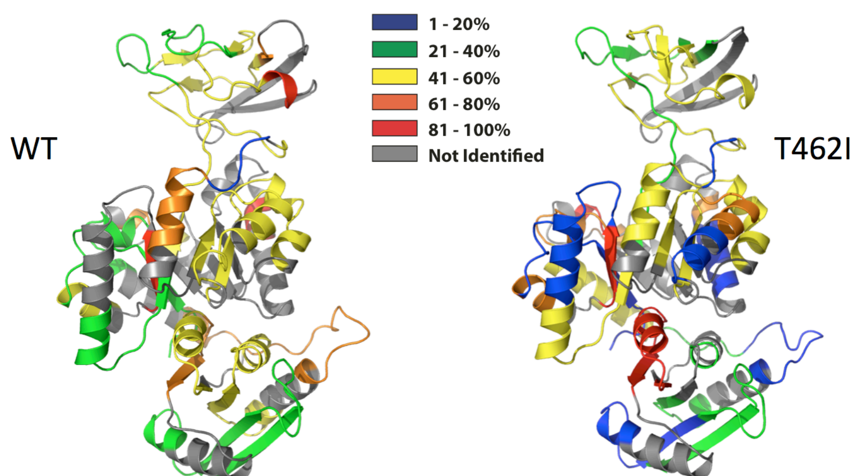

Figure 10. Peptide hydrogen/deuterium exchange studies for the pyruvate kinase enzymes demonstrate that the mutations cause a change in protein dynamics. The colours indicate the percentage deuterium uptake after 2 s (plateau region in Figure 9). Note that the pattern of uptake is different between the two proteins, demonstrating that the dynamics is different.

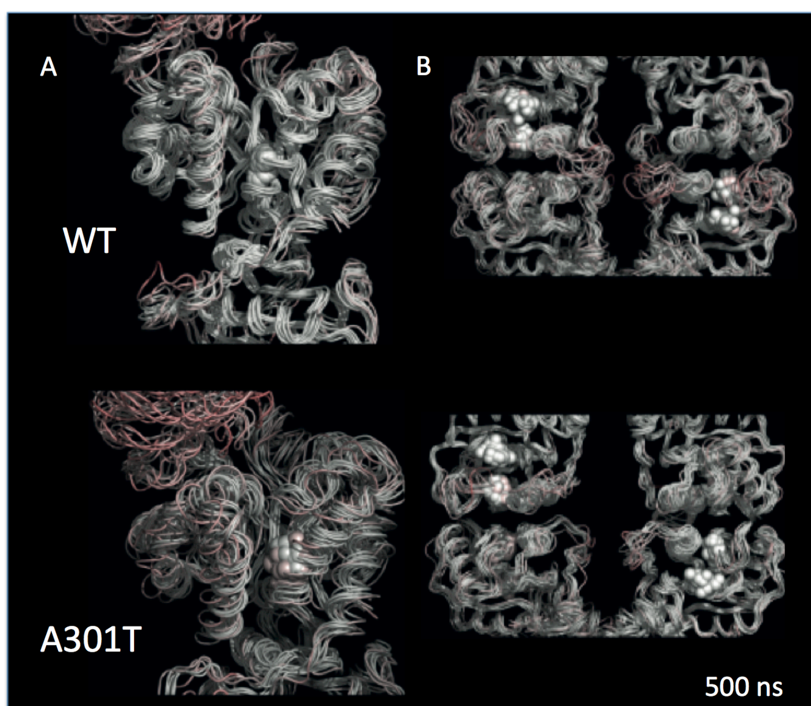

Figure 11. Molecular dynamic simulations (500 ns) of the wild type and A301T pyruvate kinase enzymes demonstrate differences in protein dynamics. In this figure, the active site domain $(\alpha/\beta)_8$-barrel (A) and allosteric binding domain (B) illustrate broad changes in dynamics. The $(\alpha/\beta)_8$-barrel of the wild type is considerably more stable than that of the A301T mutant, whereas the allosteric binding domain is more stable in the wild-type protein compared to the mutant enzyme.

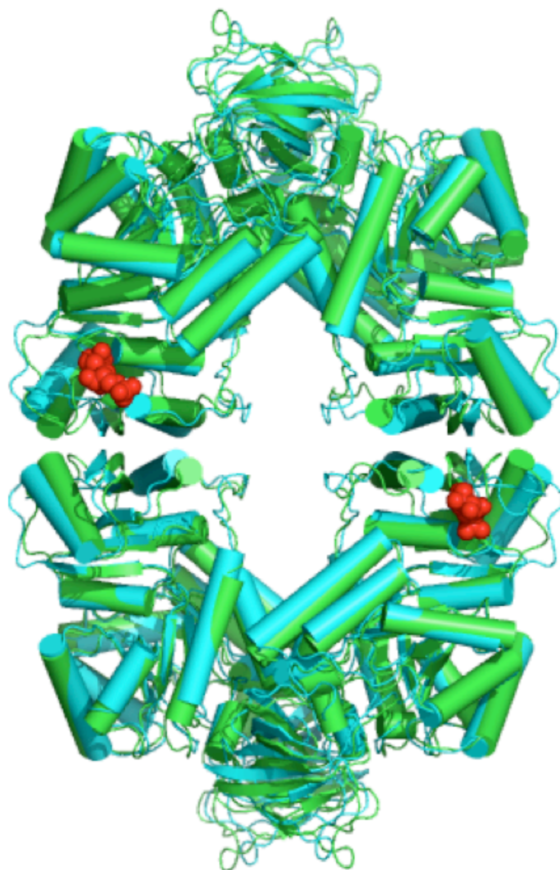

Figure 12. Overlay of the A301S pyruvate kinase with FBP (blue) and without FBP (green) showing the change in conformation that accompanies FBP binding (FBP shown as red spheres). From these data, we are able to examine the mechanism of FBP binding and ‘see’ how binding changes the conformation of the active site. Consistent with our hydrogen/deuterium exchange studies, loops at the active site (N-terminal of the $(\alpha/\beta)_8$-barrel) have higher B-factors when FBP is bound, suggesting that they are more dynamic.

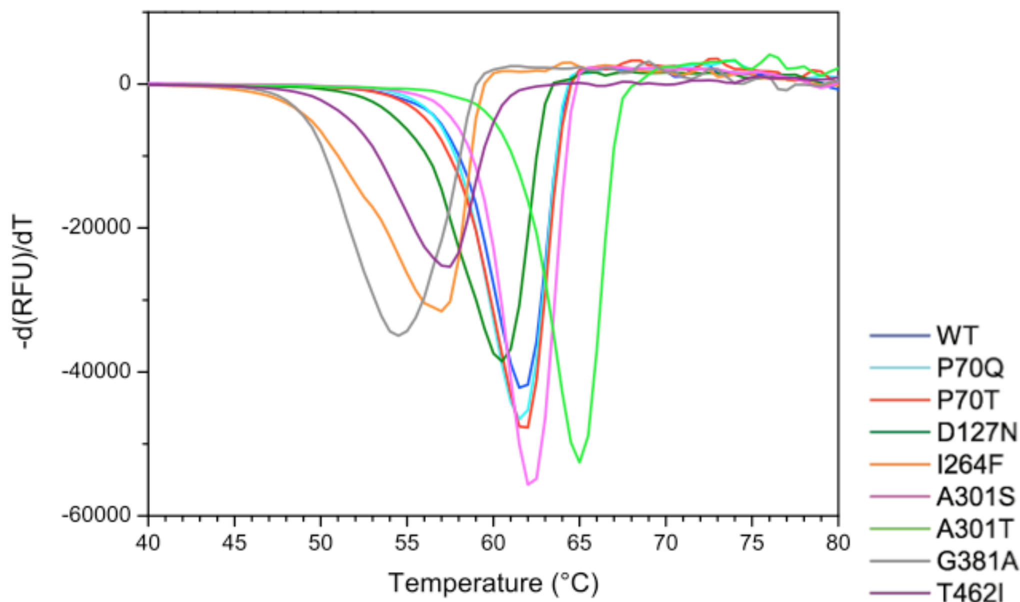

Figure 13. 1-Anilino-8-naphthalene sulfonate (ANS) binding assays demonstrate that the evolved enzymes are approximately equal in stability when compared to the wild-type enzyme (blue). While there is some changes, e.g. G381A (grey) has a lower melting temperature (54 ^{o}C) as compared to the wild-type enzyme (blue, 62 ^{o}C), overall the mutations do not consistently alter the T_m (i.e. all lower or all higher).

Question 3: **Do the adaptive pyruvate kinase mutations have secondary effects?**

Summary:

Even when the fitness effect of an adaptive mutation can be estimated, it has usually not been determined *how* the mutation causes the fitness effect that it does. We previously demonstrated that independently selected adaptive mutations in *pykF* conferred similar fitness effects when measured in a defined background. It is unknown, however, if these parallel mutations affect fitness through the same underlying mechanism. Although this may seem likely, biochemical studies of the evolved pyruvate kinase type 1 enzymes reveal a wide array of changes in enzyme kinetics that include increases and decreases in activity and stability relative to the wild type enzyme, so that it cannot be assumed that they have the same mechanism of action. To examine the basis of the benefit conferred by mutations in pyruvate kinase type 1, we proposed and tested a mechanistic hypothesis whereby mutations in pyruvate kinase act to increase stationary phase concentrations of the chemical PEP, which in turn facilitates more rapid uptake of glucose when cells are transferred to fresh medium. Consistent with predictions of this hypothesis: (1) *pykF* mutations tended to confer greater benefits in substrates requiring PEP for uptake than in alternative substrates (Figure 14), (2) stationary phase PEP levels are lower in the wild type strain than in strains containing any of the different evolved pyruvate kinase type 1 enzymes (Figure 15), and (3) the *pykF* mutations confer most of their benefit through shortening the lag time inherent in each daily growth cycle, not by increasing cell growth rate (Figure 16). Our results suggest that, while parallel mutations change the *in vitro* biochemical properties of pyruvate kinase type 1 in a range of different ways, the net effect of these changes is to confer similar adaptive changes on cell metabolism.

For extended details **see Fen Peng’s PhD thesis, Chapters 3 and 4.**

- ***Aim 6)*** *Determine the fitness of evolved pyk*F *alleles in novel resources.* **Achieved**

Detailed Results:

PEP is not only the substrate of pyruvate kinase type 1, but is also used as a source of energy to drive the phosphotransferase system (PTS), a system required for the transfer of some chemicals into the cell. If mutations in *pykF* affect stationary phase levels of PEP, we expect them to have a general effect on the initial translocation of PTS resources and therefore for *pykF* mutants to generally have higher fitness in PTS than non-PTS resource environments. We note that *pykF* mutations may well have pleiotropic effects that cause fitness to vary across environments, so this prediction does not imply that the mutation effects will be beneficial in all PTS and non-PTS environments. To test if mutations in *pykF* confer fitness advantages by affecting the PTS pathway, we measured the effect of one of the mutations (*pykF::*IS*150*) in five PTS and six non-PTS resources in the genetic background where it occurred and was fixed (Figure 14). We found the *pykF*::IS*150* mutation has a pleiotropic effect across environments: it is neutral in most of the PTS environments (mean ± SEM: 0.981 ± 0.015), but has different deleterious effect in most of the non-PTS environments (mean ± SEM: 0.877 ± 0.022). A two-way ANOVA with environment nested within resource type (PTS or non-PTS) found a significant difference between PTS and non-PTS resources in affecting fitness of the mutation ($F_{1,32}$ = 302.9, P < 0.001). This result is consistent with the hypothesis that *pykF* mutations tended to confer greater fitness in environments requiring PEP for uptake than in alternative substrates.

Additional work:

The effect of a mutation may depend on its genetic background (epistasis), the environment (pleiotropy), or both. However, few empirical studies have investigated the joint influence of both factors in the same experiment. Those that have, usually focused on randomly generated mutations, which may behave differently than beneficial mutations selected because they improve organism adaptation in a specific genetic and environmental context. We measured the fitness effect conferred by the beneficial *pykF* deletion mutation (Δ*pykF*) in 23 natural isolates of *E. coli* in five different resource environments (Figure 17). We found that the genetic background, the environment, and the interaction between the genetic background and the environment, all have significant effects on the fitness effect of the mutation, with genetic background being the most important in determining fitness. In agreement with the diminishing returns epistasis found in previous experiments, a negative relationship between the maximum growth rate of the recipient strains and the fitness effect of Δ*pykF* were found in all five environments (Figure 18), although only in one environment was this relationship significant. Our results suggest that the effect of beneficial mutations will be difficult to predict, depending on specific interactions with genotype and environment. Nevertheless, we find support for a growing body of research predicting a qualitative relationship between mutation effects and initial fitness of a progenitor strain, predicting that the contribution of a mutation will be smaller when added to fitter progenitors. (See Fen Peng’s PhD Thesis Chapter 4 for details.)

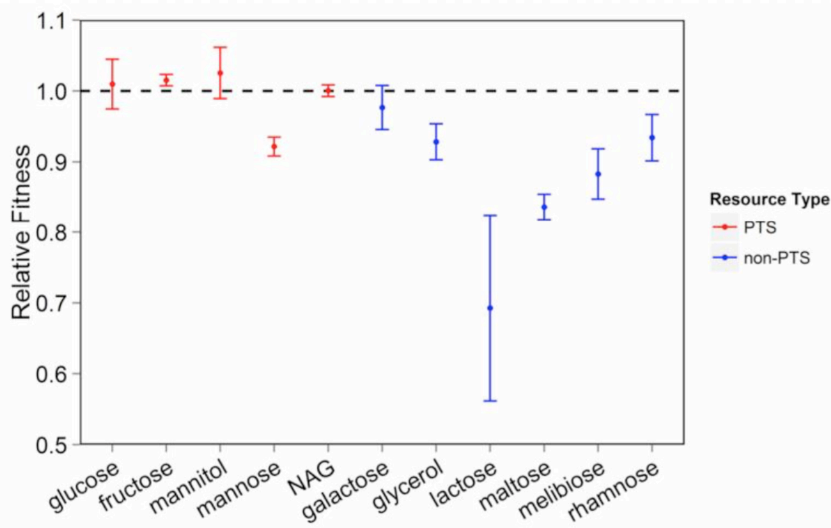

Figure 14. Fitness effect of *pykF*::IS*150* mutation in 11 resources. Five are PTS resources (red): glucose, fructose, mannitol, mannose, and NAG; six are non-PTS resources (blue): galactose, glycerol, lactose, maltose, melibiose, and rhamnose. The neutral effect of *pykF*::IS*150* in glucose seems contradictory with the previous 6% benefit shown in Figure 1, because the fitness assays here were performed in 96-well plates (see Materials and methods) rather than flasks.

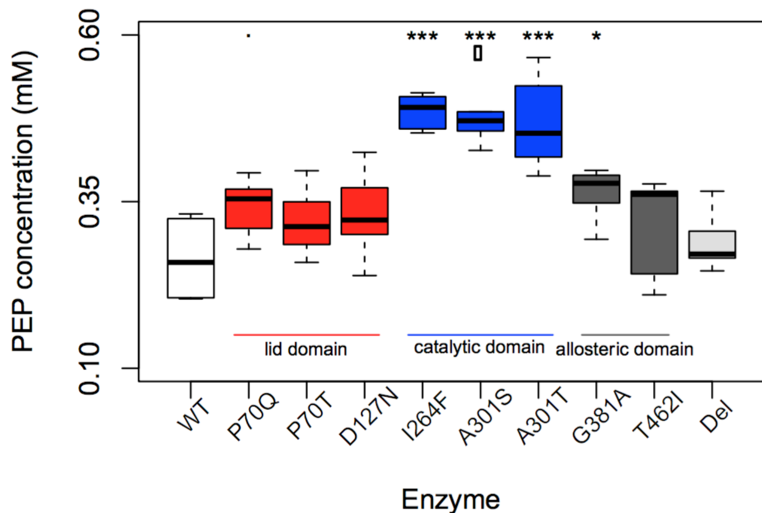

Figure 15. Box and whisker plot indicating PEP concentration of otherwise isogenic strains expressing the indicated PykF enzyme ('Del' is the deletion allele). Strains were sampled following 24 hours of growth in the evolution environment. Boxes indicate first and third quartiles, the central line indicates the median, and whiskers indicate 95% confidence intervals of the median. Symbols at top of panel reflect results of Dunnett's test comparing strains with evolved enzymes against the WT enzyme strain: "***" <0.001, "*" 0.05, " " 0.1. Results are from six independent replicate measurements of each strain.

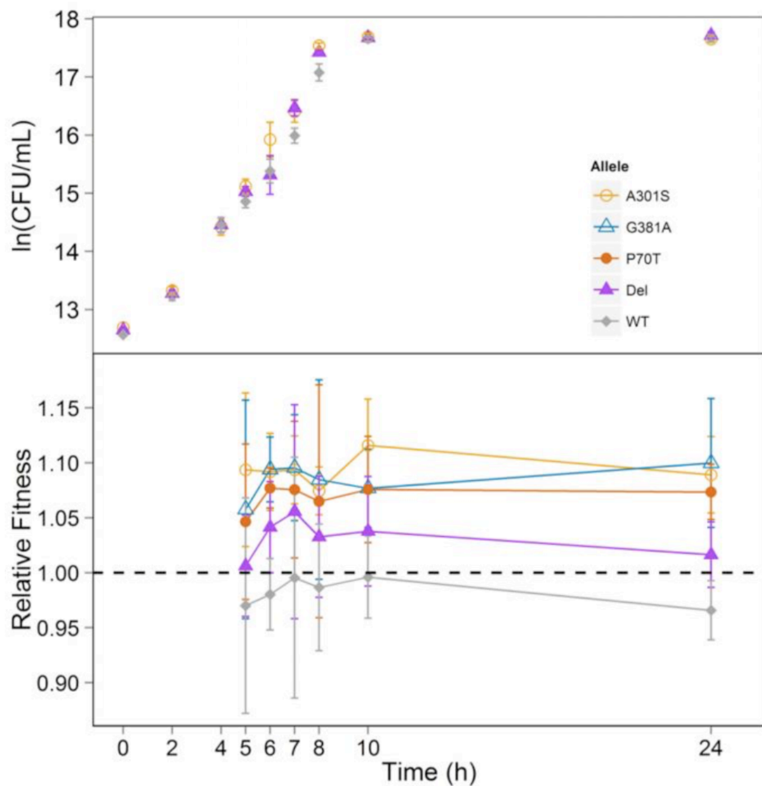

Figure 16. Growth and fitness measurements of the ancestral strain and derivatives encoding one of four different *pykF* alleles over 24 hours. **s**Upper panel shows the growth measurement for ancestral strains with WT, Del, and A301S allele, and error bars indicate 95% confidence intervals of independent CFU estimates (n=6). Lower panel shows the fitness effects of *pykF* alleles at different time points. Symbols and error bars represent the mean and 95% confidence intervals of four independent measurements.

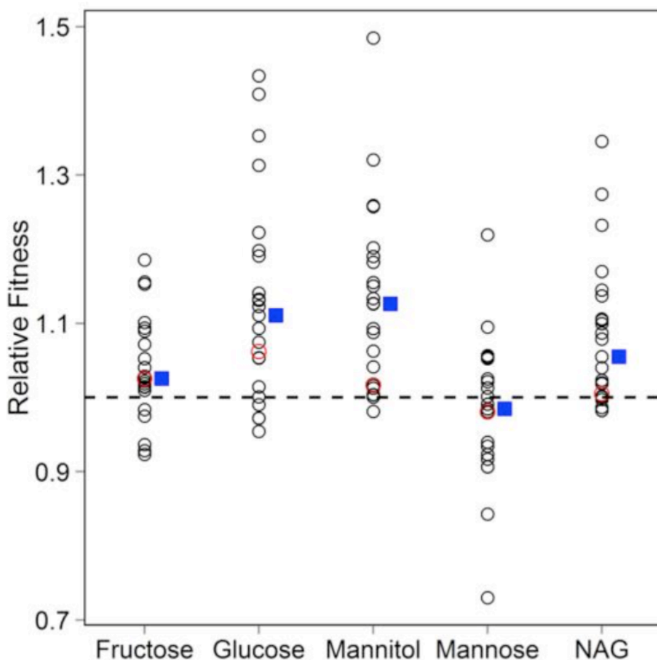

Figure 17. The Δ*pykF* mutation was added to the 23 genetic backgrounds and their fitness measured relative to the original progenitor strain in each of five PTS resource environments. Hollow symbols represent the mean fitness of the Δ*pykF* mutation in each strain based on three or four independent estimates. Red hollows indicate the fitness of Δ*pykF* in the genetic background on which it arose and fixed. Blue squares represent the grand mean fitness in each environment.

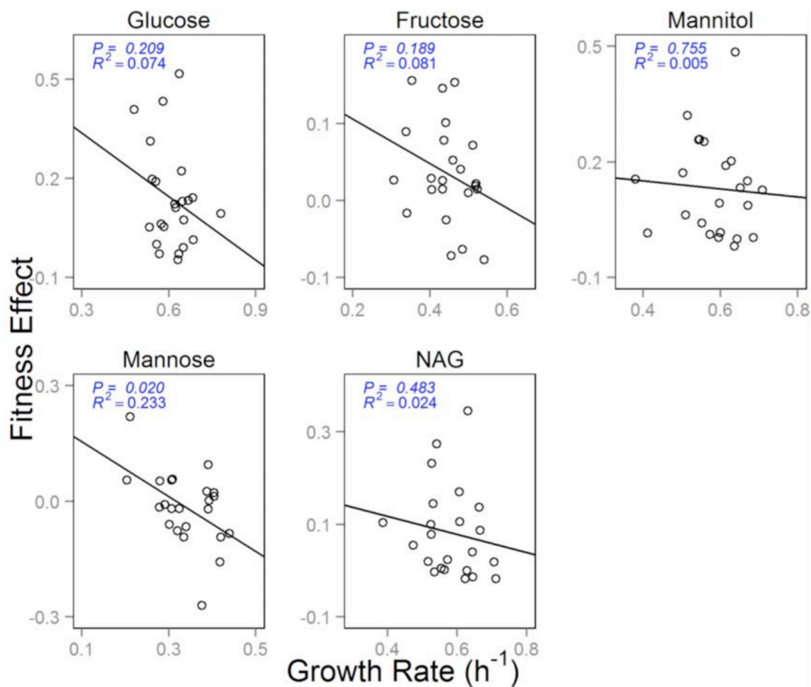

Figure 18. Relationships between maximum growth rate and relative fitness of adding the Δ*pykF* mutation to the 23 genotypes in five PTS resource environments. Hollows represent the mean of maximum growth rate of the 23 genotypes (3 ≤ n ≤ 32), and the mean of fitness of the Δ*pykF* mutation in each genotype (n = 3 or 4). Lines show linear regression with *P* value and R^2 calculated in a Pearson correlation shown for each graph. Spearman non-parametric correlations were also performed, and they did not affect conclusions of significance.

Impact

Our program provides a uniquely detailed functional understanding of how evolution by natural selection occurs at the molecular level. Many studies have described the genetic and physiological changes that occur during evolution. *Our work is the first to link a quantified fitness advantage to specific adaptive mutations* via *changes in enzyme structure (dynamics), function and metabolic consequences in the context of the E. coli long-term evolution experiment.*

The data sets generated by our work can be analyzed in the context of a wide range of fields (e.g. physiology, biochemistry and bioinformatics). Such multidisciplinary analyses generate a level of empirical detail that will be invaluable to the goal of developing a mechanistic understanding and prediction of the process of adaptation.

Independently, this work also provides unique insights into the mechanism of pyruvate kinase function. In particular, we have been able to delineate how FBP binding alters the structure and dynamics of pyruvate kinase to activate the enzyme. These data has been lacking for this enzyme until now.

Future research

We envisage two future programs of work:

1) We have demonstrated that the adaptive changes in pyruvate kinase are mediated through changes in protein dynamics. Is this a general phenomena? The gene *spoT* is also found to contain adaptive mutations in most of the 12 populations. Upon starvation, bacteria slow down the physiological processes for cell growth and speed up the processes to overcome nutrient deficiency. This stringent response is regulated by the RelA and SpoT enzymes, which catalyze the synthesis and degradation of a signaling alarmone, ppGpp. Interestingly, the SpoT enzyme of *E. coli* comprises both a ppGppase and GDP synthetase, thus regulating the ppGpp levels in the cell. To test whether the adaptive mutations in SpoT also alter protein dynamics, we first require a thorough structural and biochemical understanding of the SpoT enzyme, which, unlike pyruvate kinase, is currently lacking. We have initiated such studies of the SpoT enzyme in an effort to understand how this enzyme functions and why it is also target for adaptive evolution.

2) During the course of our program, we became curious about the nature of the two pyruvate kinase deletion mutations that are found in the long-term *E. coli* evolution experiment. Interestingly, the gene deletions result in a truncated $(\alpha/\beta)_8$-barrel protein – the fold of the catalytic domain – such that the resulting protein constitutes a half and quarter barrel. To date, half $(\alpha/\beta)_8$-barrel folds have only been engineered and have never been observed in nature as an adaptive change. Moreover, the quarter barrel has been hypothesized, but never successfully observed (engineered or otherwise). We have demonstrated that both truncations can be overexpressed and are soluble! We are currently working to purify these proteins. This work has the potential to significantly add to the story of how $(\alpha/\beta)_8$-barrels evolved.