

**Understanding the mechanisms by which *E. coli* can adapt to disruption of the pathway for  
the synthesis of pyridoxal-5'-phosphate**

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## Abstract

*Escherichia coli* possesses a remarkable ability to adapt to new environments and overcome disruption of synthetic pathways. In this thesis, I show that *E. coli* with a deleted *pdxB* gene is able to bypass a disruption in the pyridoxal-5'-phosphate (PLP) synthesis pathway. The  $\Delta pdxB$  strain cannot synthesize its own PLP via the traditional pathway, so it must create a new pathway to synthesize this essential vitamin. One way the  $\Delta pdxB$  strain adapts is via serendipitous pathways that emerge as a direct result of mutations in PLP-depleted environments. These pathways appear when the strain is grown on both solid and liquid media.

Mutations restoring PLP synthesis occurred in the  $\Delta pdxB$  strain when plated on solid M9 minimal medium supplemented with 0.2% (w/v) glucose or glycerol, or when supplemented with over 2% (w/v) pyruvate, succinate, and acetate. The  $\Delta pdxB$  strain adapted in liquid M9/glycerol, but only in favorable conditions. These conditions, such as when the strain was supplemented with D-alanine or after the strain synthesized a proteome tailored to glycerol, allow survival of the bacterium by helping minimize the detriment of its weak cell wall. The longer the bacteria can survive, the more opportunity there is for mutations to appear. Whole-genome sequences of adapted strains were analyzed using *breseq*, revealing that the adapted  $\Delta pdxB$  strains acquired mutations, which are assumed to result in the synthesis of PLP. It is currently unclear the exact effect these mutations have and whether or not they are all involved in a serendipitous pathway.

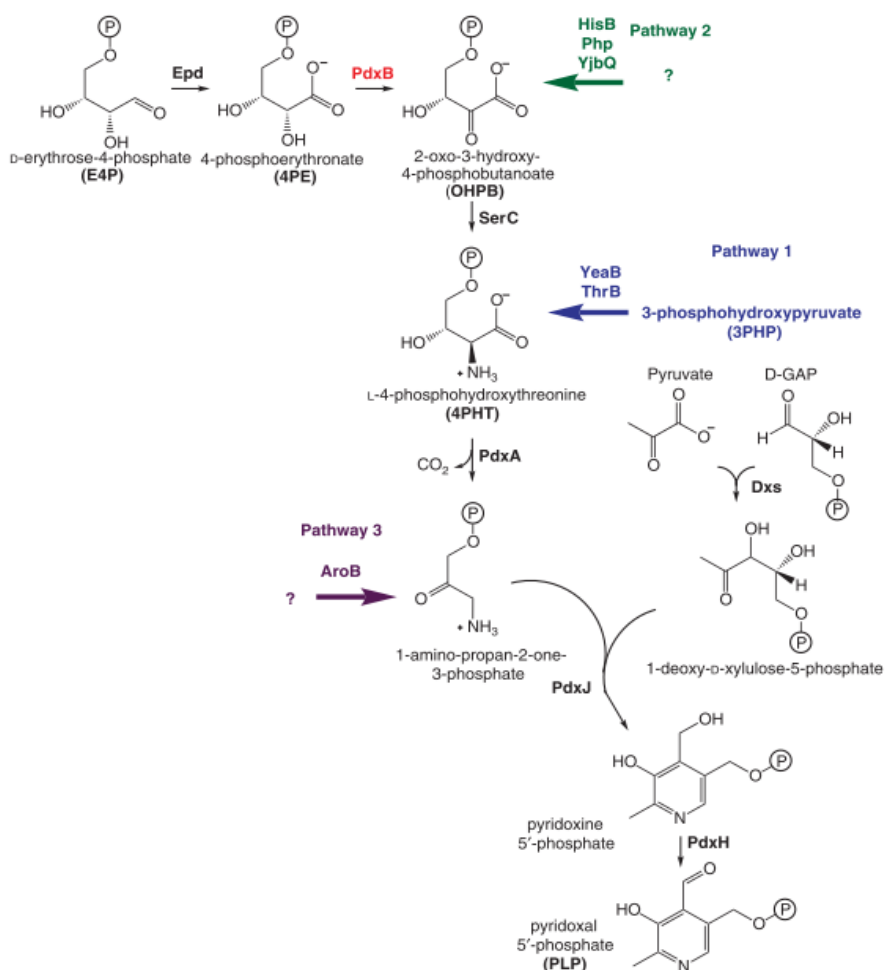
## Chapter 1

### Introduction

*Escherichia coli* has shown a great ability to adapt to new circumstances.<sup>1-3</sup> One of the remarkable ways they can adapt is by bypassing a enzymatic step in a biosynthesis pathway when the gene encoding the enzyme is mutated or deleted. At one point in time, it seemed unlikely that a bacterium could survive without the enzyme encoded by the mutated or deleted gene. The bacterium would die due to a lack of the enzyme's vital metabolite. This assumes that no other enzyme would be able to catalyze the missing reaction due to the specificity of the enzyme for its substrate. This "lock and key" model of the enzyme-substrate complex fails to describe the entire story. Emerging research is remodeling this idea. Researchers are revealing enzymes' abilities to catalyze multiple reactions, albeit not always at physiologically relevant levels. These activities are considered promiscuous activities.<sup>4</sup> *E. coli* can use these promiscuous activities to patch together new serendipitous pathways if the canonical pathway is disrupted.<sup>5</sup> These new serendipitous pathways can help researchers further understand how enzymatic pathways evolved. If we can understand how these pathways emerge, we may be able to create new novel enzymatic pathways that address new problems.

This present study builds upon previous work conducted in the Copley lab at the University of Colorado involving the promiscuous activities of enzymes and new serendipitous pathways created by patching together promiscuous activities.<sup>6</sup> In particular, the Copley lab studies serendipitous pathways involved in pyridoxal-5'-phosphate (PLP) synthesis.

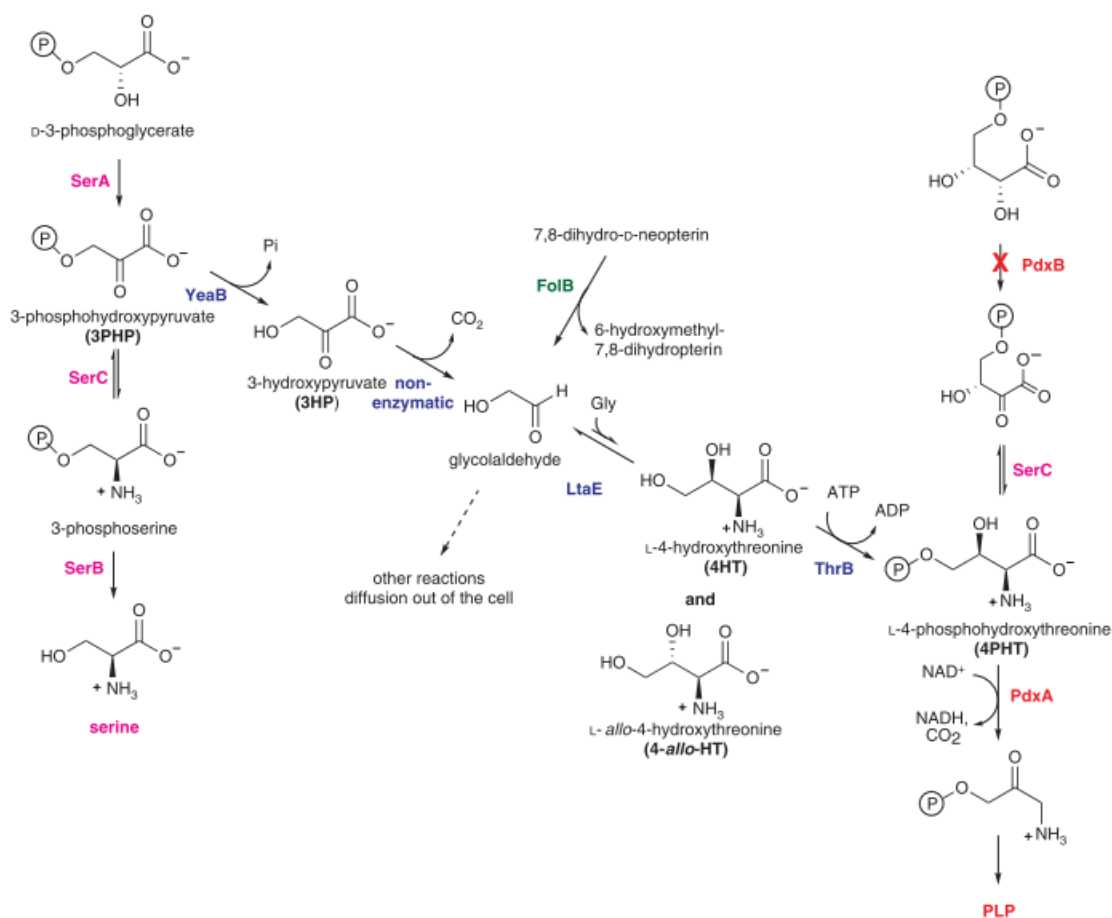
Essential for all organisms, PLP serves as a cofactor for many different enzymatic reactions,<sup>7</sup> making it a crucial molecule for cell growth. PLP is also required in very low concentrations, so even an inefficient serendipitous pathway could make enough PLP for the cell to grow. The Enzyme Commission states that 4% of all enzymes are PLP-dependent.<sup>8</sup> PLP-dependent enzymes are encoded by 1.5% of bacterial genes, accounting for over 140 distinct enzymatic activities.<sup>9</sup> As a result of the numerous cellular processes that involve PLP in bacteria, such as transamination reactions in amino acid synthesis<sup>8</sup> and racemization of amino acids,<sup>10</sup> a



**Figure 1.1** From Kim *et al.*, (2010). Pyridoxal-5'-phosphate synthesis pathway showing three serendipitous pathways that feed intermediates downstream of *pdxB*

disruption in the synthesis pathway forces the bacteria to overcome the disruption if they are to survive.

Some serendipitous pathways allow *E. coli* to bypass a disruption in the PLP synthesis pathway by producing substrates downstream of the disruption, as shown in Figure 1.1.<sup>5</sup> In these experiments, the PLP synthesis pathway was artificially disrupted via knocking out the *pdxB* gene<sup>11</sup> that encodes the enzyme 4-phosphoerythronate dehydrogenase (PdxB). This enzyme catalyzes the second reaction in PLP synthesis. The  $\Delta$ *pdxB* strain cannot synthesize PLP via the normal pathway, so it must find an alternative way to synthesize PLP.



**Figure 1.2** From Kim *et al.*, (2010). A fully characterized Serendipitous Pathway 1 shows the conversion of 3PHP through four steps to 4PHT, an intermediate in PLP synthesis.

One way in which *E. coli* is able to bypass a disruption in the PLP synthesis pathway is through serendipitous pathways.<sup>5</sup> Kim *et al*<sup>5</sup> describes a serendipitous pathway in detail (Figure 1.2). This pathway pulls an intermediate from serine biosynthesis and in four steps produces L-4-phosphohydroxythreonine (4PHT), a PLP intermediate. Increasing the concentration of 3-phosphohydroxypyruvate (3PHP) increases flux through this pathway, allowing growth of the bacterium.<sup>5</sup> Additionally, increased levels of any of the three enzymes used in this serendipitous pathway or an increase in their promiscuous activity will increase flux through this pathway. However, only a mutation will allow a serendipitous pathway to produce enough PLP to sustain growth of the  $\Delta pdxB$  strain, thus becoming physiologically relevant.

The formation of these serendipitous pathways depends on many different factors, including environmental conditions. The cell responds to a new environment (for example, growing on a different carbon source) by expressing a new suite of enzymes and altering the concentrations of metabolites present in the cell.<sup>6,12</sup> In this case, glucose, glycerol, pyruvate, succinate, and acetate are interesting carbon sources because they are metabolized in different steps of glycolysis and the TCA cycle. Glucose can quickly be metabolized to acetate. Glycerol can feed directly into glycolysis. Pyruvate, succinate, and acetate undergo gluconeogenesis and respiration.<sup>13</sup> These different modes of metabolizing the carbon source will result in different gene expression and a different metabolic network.<sup>13,14</sup>

Changing the carbon source the bacterium metabolizes would alter the concentrations of metabolites present in the cell.<sup>15</sup> If the concentration of a metabolite that feeds into a serendipitous pathway increases enough, flux through the serendipitous pathway might increase to physiologically relevant levels. Another way a serendipitous pathway could emerge is if an enzyme with a promiscuous activity becomes mutated. If this mutation is in an enzyme



that is expressed on a specific carbon source and the mutation causes the enzyme's promiscuous activity to reach physiologically relevant levels, it will increase flux down a serendipitous pathway and allow growth.

In my honors thesis work, I searched for ways in which the  $\Delta pdxB$  strain restores PLP synthesis. The  $\Delta pdxB$  strain can mutate under PLP-starved conditions by using glucose, glycerol, pyruvate, succinate, or acetate as a carbon source. Bacteria that grow under these altered conditions are deemed pseudorevertants, as they "revert" back to their original phenotypes without a reversion in genotype. Mutations a pseudorevertant acquires may lead to a bacterium's ability to synthesize PLP through a serendipitous pathway. Additional mutations might increase the cell's fitness after a serendipitous pathway emerges. Therefore, four hypotheses need to be considered: 1) the mutation increases PLP synthesis through a serendipitous pathway; 2) the mutation compensates for deleterious side-effects of mutations that increase flux through a serendipitous pathway; 3) the mutation is simply reducing the toxic effect of a serendipitous pathway intermediate but not increasing flux through the pathway; 4) the mutation reduces the bacterium's need for PLP, allowing it to survive with less PLP.

My first objective was to determine specific conditions in which the  $\Delta pdxB$  strain can grow on solid medium. We can better understand potential ways the  $\Delta pdxB$  strain restores PLP synthesis by observing varying conditions that facilitate growth of pseudorevertants. The second objective is a long-term adaptation experiment in liquid medium. Dr. Juhan Kim in the Copley lab showed that the  $\Delta pdxB$  strain can adapt and overcome PLP starvation after 200 generations when grown in M9/glucose. The adapted strains acquire mutations which can be explained by one of the four hypotheses. Adapting the  $\Delta pdxB$  strain to survive on other carbon

sources might reveal different mutations that allow PLP synthesis. Patterns could emerge when comparing the mutations of adapted strains on various carbon sources. These patterns could paint a picture of the types of mutations that lead to PLP synthesis.

My project was significant because it looks at ways that bacteria grown on different carbon sources adapt. I explored the mutations acquired by these adapted strains in an attempt to correlate the mutations with the rise of serendipitous pathways. Recognizing a relationship between the external growth conditions and the serendipitous pathways that emerge will give a better understanding of the evolution of enzymatic pathways.

## Chapter 2

### Adaptation of the $\Delta pdxB$ strain in the presence of different carbon sources

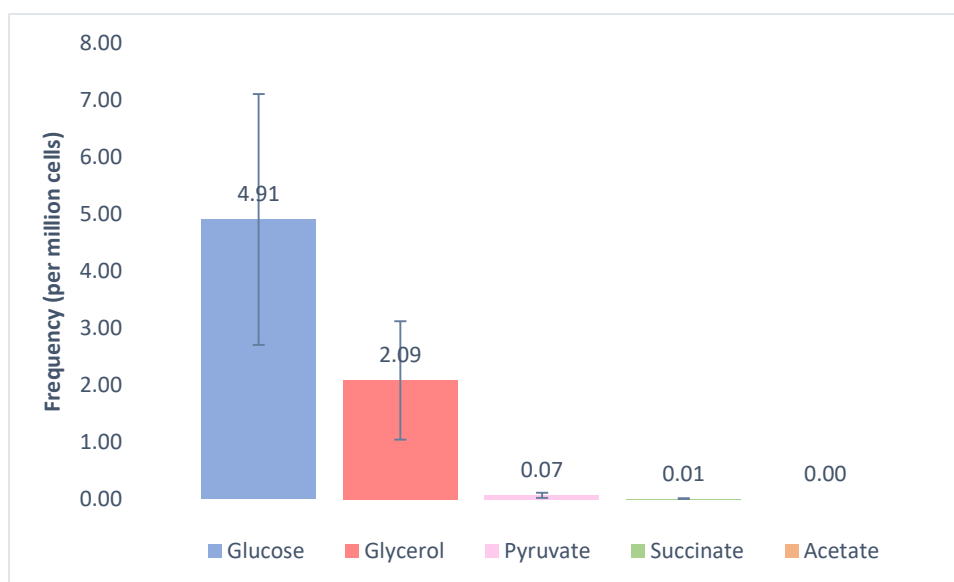
The  $\Delta pdxB$  strain cannot synthesize PLP via its normal PLP synthesis route due to the deletion of the *pdxB* gene; therefore, the  $\Delta pdxB$  strain must find an alternate route to synthesize PLP. After growing the  $\Delta pdxB$  strain on M9 minimal media using various carbon sources, we saw that providing the strain with carbon sources able to be metabolized by glycolysis better facilitated adaptation on plates. The bacteria under these favorable conditions could adapt and synthesize PLP via a serendipitous pathway.

## Results

*Pseudorevertants appear when the  $\Delta pdxB$  strain is grown on M9 supplemented with glucose, glycerol, pyruvate, or succinate*

The  $\Delta pdxB$  strain was grown to an  $OD_{600}$  of 0.2 – 0.6 in LB and washed in PBS to remove excess PLP from the media. The washed cells were spread onto M9/carbon plates containing glucose, glycerol, pyruvate, succinate, or acetate, and allowed upwards of 14 days of incubation at 37 °C. Pseudorevertant colonies were counted and compared to the total number of cells plated, determined by serial dilutions of the washed cells on LB/kanamycin plates (Figure 2.1). We hypothesized that the frequency of pseudorevertants colonies that appeared would vary between the carbon sources due to the different oxidation states of the carbon sources and the different metabolites and enzymes present.

Plates containing M9/1xglucose (0.2% w/v glucose) consistently yielded the greatest frequency of pseudorevertants (Figure 2.1) and had pseudorevertant colonies that reached the largest size (greater than 2 mm) (Figure 2.3). Many colonies on these plates had varying sizes and morphologies. Some colonies appeared after 3-5 days and grew to 2 mm in diameter while others appeared after a week of incubation and never reached larger than 0.5 mm in diameter. All colonies appeared white in color. M9/glycerol yielded the second greatest frequency of

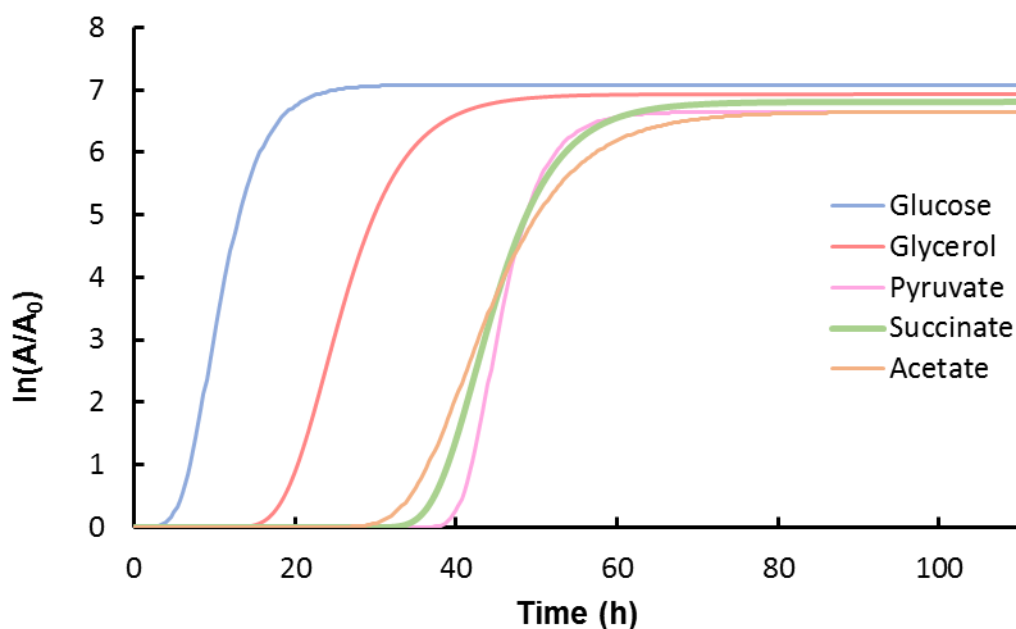


**Figure 2.1** Frequency of pseudoreversion of the  $\Delta pdxB$  strain when grown on M9 plates supplemented with one of five carbon sources.

pseudorevertants at approximately half the rate of M9/glucose (Figure 2.1). Pseudorevertant colonies appear on glycerol at a more consistent rate and most grew to a consistent size of approximately 1 mm in diameter.

Wild-type *E. coli* were grown in LB and transferred to M9 containing one of the five carbon sources. Pyruvate, succinate, and acetate had longer lag times as compared to glucose and glycerol, indicating how much time might be required for the bacteria to induce expression of all the enzymes needed to grow on the carbon sources (Figure 2.2). These carbon sources differ from glucose and glycerol because they are metabolized via respiration or undergo

gluconeogenesis.<sup>16</sup> Succinate and pyruvate facilitated pseudorevertant colony sizes of a maximum of 0.5 mm while acetate only showed colonies that appeared much smaller than 0.5 mm. These tiny pinprick colonies were classified as background colonies and could be seen most prevalently on M9/succinate and M9/acetate plates.



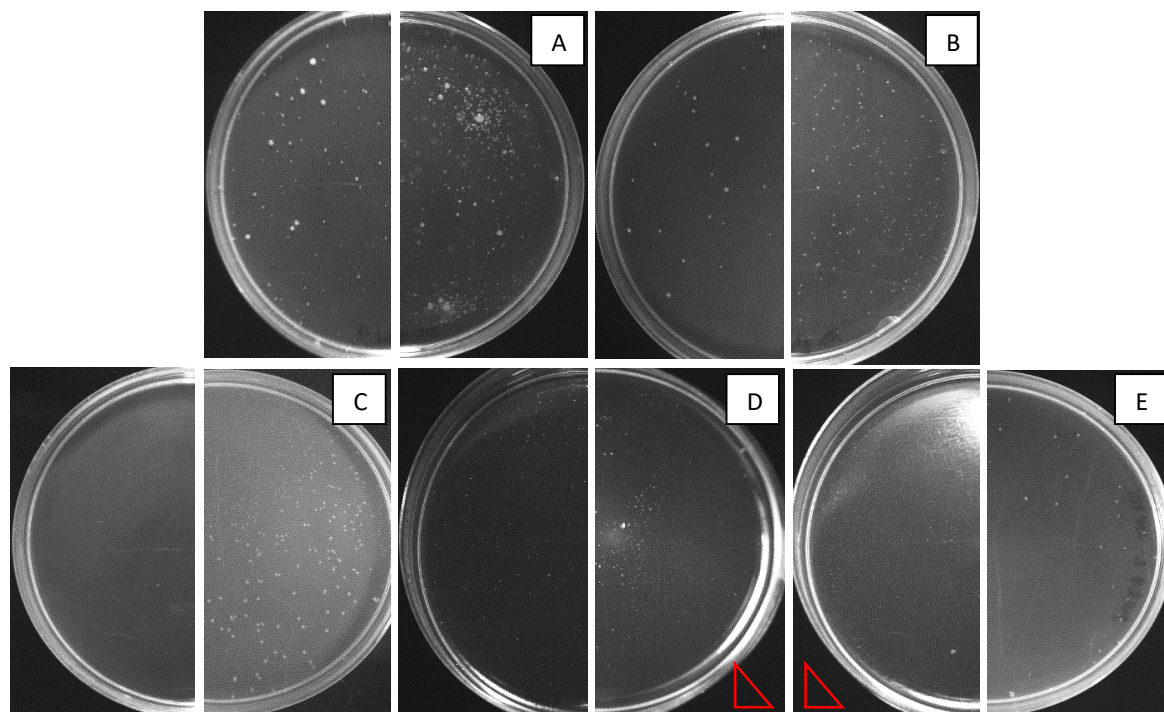
**Figure 2.2** Supplementation of different carbon sources results in varying lag times for wild type *E. coli*. Cells were washed before being inoculated into M9/1xcarbon.  $A = OD_{600}$

Background colonies may appear due to a low flux serendipitous pathway but cannot sustain exponential growth. To determine whether these background colonies should be counted as true pseudorevertants, a background colony from an M9/succinate plate was restreaked onto a fresh M9/succinate plate and allowed to grow for 5-7 days. Out of the 13 colonies tested, only four had any signs of growth. It is possible that some of these background colonies are indeed pseudorevertants. No potential pseudorevertants were re-tested on plates containing acetate as these colonies were even smaller than the succinate colonies tested. Even if these colonies were pseudorevertants, their growth would be difficult to distinguish from

background growth due to the similarities between the growth and would result in many false positive pseudorevertants.

More pseudorevertants were observed at higher concentrations of each carbon source

Previous experiments were conducted by supplementing minimal media with glucose, glycerol, pyruvate, succinate, or acetate at 0.2%, 0.2%, 0.24%, 0.45%, 0.27% (w/v) respectively. These concentrations, which were classified as 1x, keep the molar concentration of carbon constant across the carbon sources. These experiments were repeated using ten times (10x) the amount of carbon source. After incubation at 37 °C for seven days, a markedly different number of pseudorevertants grew.



**Figure 2.3** Comparison between 1x/carbon (left) and 10x/carbon (right). Plates with red triangle indicate the plate was imaged at an angle to better refract light and highlight colonies. All images taken after 12 days of growth at 37 °C. (A) glucose (B) glycerol (C) pyruvate (D) succinate (E) acetate

Pseudorevertants appeared at a much higher frequency on 10x glucose, glycerol, pyruvate, and succinate as compared to the frequency at 1x carbon, and for the first time, pseudorevertants grew on acetate. Cells plated on M9/1x carbon were not diluted due to the low frequency of pseudoreversion. However, cells plated on M9/10x carbon were diluted 100 fold (glucose and acetate) and 10,000 fold (glycerol, pyruvate, and succinate) as a direct result of the increased frequency of pseudoreversion (Figure 2.3). The colonies grown on glucose were irregular in size, varying from tiny pinpricks to over 2 mm in diameter after 5-7 days. Pseudorevertants that arose on glycerol and pyruvate had much more consistent sized colonies, all ranging between 0.5 mm and 1.0 mm in diameter. Interestingly, acetate pseudorevertants had consistent colony morphology but appeared at a much lower rate than glucose and glycerol pseudorevertants. Succinate yielded background colonies except for the very rare pseudorevertant colony that managed to grow to 0.5 mm in diameter. All of the carbon sources had background colonies to varying degrees.

## Discussion

The  $\Delta pdxB$  strain shows a remarkable ability to adapt in environments that do not provide pyridoxine, which can be scavenged and converted to PLP via a salvage pathway. The data presented here show that mutations can allow the  $\Delta pdxB$  strain to grow on multiple different carbon sources. Glucose is quickly metabolized by *E. coli* when it is converted into acetate. Glycerol provides similar amount of energy as compared to glucose when fermented, but at a slightly slower rate as evidenced by slower doubling times.<sup>17,18</sup> Acetate, succinate, and pyruvate provide less immediate energy for growth of the  $\Delta pdxB$  strain as evidenced by an

increased lag phase (Figure 2.2). The frequency of pseudorevertants parallels the method of which the  $\Delta pdxB$  strain can utilize the carbon source. Carbon sources that can be metabolized in glycolysis facilitate an increased frequency of pseudorevertants as compared to carbon sources that are metabolized in the TCA cycle and undergo gluconeogenesis (Figure 2.1). These glycolysis carbon sources may be synthesizing large quantities of enzymes or metabolites specific for glycolysis. Any one of these enzymes or metabolites could be utilized in a serendipitous pathway.

Pyruvate, succinate, and acetate allow few pseudorevertants at 1x concentration. However, when the  $\Delta pdxB$  strain was plated on these carbon sources at 10x concentration, the frequency of pseudoreversion increased tremendously. This result implied a serendipitous pathway may reach physiologically relevant levels only when a mutation appears in conjunction with an increased concentration of one of the metabolites or enzymes present in the  $\Delta pdxB$  strain.

One metabolite that might increase flux through a serendipitous pathway is pyruvate, albeit somewhat indirectly. Increased concentration of pyruvate, which can be converted to 1-deoxy-D-xylulose 5-phosphate by *dxs*, may increase flux through the lower half of PLP synthesis (Figure 1.1). Increased levels of 1-deoxy-D-xylulose 5-phosphate would increase the synthesis of pyridoxine-5'-phosphate. This reaction also consumes 1-amino-propan-2-one-3-phosphate, which in turn would lower the concentration of 4PHT and 2-oxo-3-hydroxy-4-phosphobutanoate (OHPB). Flux through a serendipitous pathway that synthesizes one of these PLP intermediates would be increased as the products of the pathway are consumed. Only if a mutation also increased flux through the serendipitous pathway would the pathway become



physiologically relevant. In this example, the metabolite pyruvate does not directly feed into a serendipitous pathway but results in increased flux through the pathway.

## Methods

### Strains

The parent  $\Delta pdxB$  strain is the *E. coli* BW25113 strain with the *pdxB* gene knocked out and replaced by a kanamycin resistance gene from the Keio collection.<sup>11</sup>

### Chemicals

The carbon sources used in these experiments were sodium acetate trihydrous, granular (Mallinckrodt Chemicals); sodium succinate dibasic hexahydrate (Sigma); D-glucose (Fisher); glycerol (Fisher); sodium pyruvate (Sigma). Compounds to create M9 salts and phosphate buffer saline were obtained from Fisher. Kanamycin and Luria Broth (LB) were obtained from Research Products International Corp. Luria agar (Miller's LB Agar) was purchased from Alpha Biosciences.

### M9 minimal media plates supplemented with a carbon source

M9 minimal media was created following the recipe provided by Sigma Aldrich. M9 plates were created with the addition of a carbon source at both 1x and 10x concentrations. The concentrations of carbon source used were to ensure equal moles of carbon added to each plate. The carbon sources and both their 1x and 10x concentrations used were glucose (0.2%

and 2.0% w/v), glycerol (0.2% and 2.0% w/v), sodium succinate (0.45% and 4.5% w/v), sodium acetate (0.27% and 2.7% w/v), and sodium pyruvate (0.24% and 2.4% w/v).<sup>19</sup>

*Plating the  $\Delta pdxB$  strain on solid M9 minimal media to obtain pseudorevertants*

Freezer stocks of the  $\Delta pdxB$  strain were streaked onto fresh agar plates containing LB with 50  $\mu\text{g/ml}$  kanamycin and grown overnight at 37 °C. A colony was then picked and inoculated into 5 ml of LB with 50  $\mu\text{g/ml}$  kanamycin and allowed to grow overnight at 37 °C. From the liquid culture, 50  $\mu\text{l}$  of cells were inoculated into 5 ml of LB/kanamycin and allowed to grow until log phase (0.4 - 0.8  $\text{OD}_{600}$ ). One ml of cells was harvested by centrifugation (16100 x g for one minute at room temperature). The cell pellet was resuspended in 700  $\mu\text{l}$  of ice-cold phosphate-buffered saline (PBS) and subsequently washed five times with 700  $\mu\text{l}$  PBS to remove pyridoxine from the residual LB medium. After washing, the cell pellet was resuspended in 500  $\mu\text{l}$  of PBS. On an M9 plate supplemented with 1x or 10x of the carbon source (M9/1xcarbon and M9/10xcarbon), 50  $\mu\text{l}$  of washed cells were spread. Plates were incubated at 37 °C for 12-14 days. Pseudorevertants were determined based on their ability to grow to a colony size of 0.5 mm or greater in diameter without coming into contact with other colonies to prevent PLP excretion from one pseudorevertant colony supplementing another non-pseudorevertant colony. Colonies under 0.5 mm in diameter required further testing to determine if they were pseudorevertants.

## Chapter 3

### Verification of pseudoreversion and growth on various carbon sources

Increasing the concentration of the carbon source added to M9 plates increased the frequency of pseudoreversion across all five carbon sources. However, the increased levels of growth begged the question as to whether or not the colonies growing are indeed pseudorevertants. By culturing the pseudorevertants onto fresh plates, it became clear which colonies were pseudorevertants by their homogenous growth across the plate. After verifying that a majority of the colonies were actual pseudorevertants, we saw that the pseudorevertants were also able to grow on other carbon sources. This observation led to the conclusion that the pseudorevertants were able to synthesize PLP on different carbon sources.

## Results

### Verification of pseudoreversion for colonies that appeared on M9/10xcarbon

To verify that pseudorevertants that arose on M9/10xcarbon (Figure 2.3) are indeed pseudorevertants, bacteria were grown on fresh M9/1xcarbon plates. As reported in Table 3.1, the pseudorevertant strains that originated on 10xglucose all showed growth when streaked onto M9/1xglucose. Sixty-six % showed significant growth with colony sizes uniformly reaching 1 mm in diameter after 8-12 days. The other 33% showed signs of growth with less robustness but still had a higher efficiency of plating (EOP) than the  $\Delta pdxB$  mutant alone. EOP is determined by comparing the total number of cells that grew on an M9/1xcarbon plate to the

total number of cells that grew on an M9/1xcarbon plate with pyridoxine, which served as the positive control. Glycerol pseudorevertants grew very well, with 85% having consistent colony sizes greater than 1 mm in diameter. The remaining 15% met the standard for being real pseudorevertants by having a consistent growth across the plate albeit reaching a smaller diameter of approximately 0.5 mm.

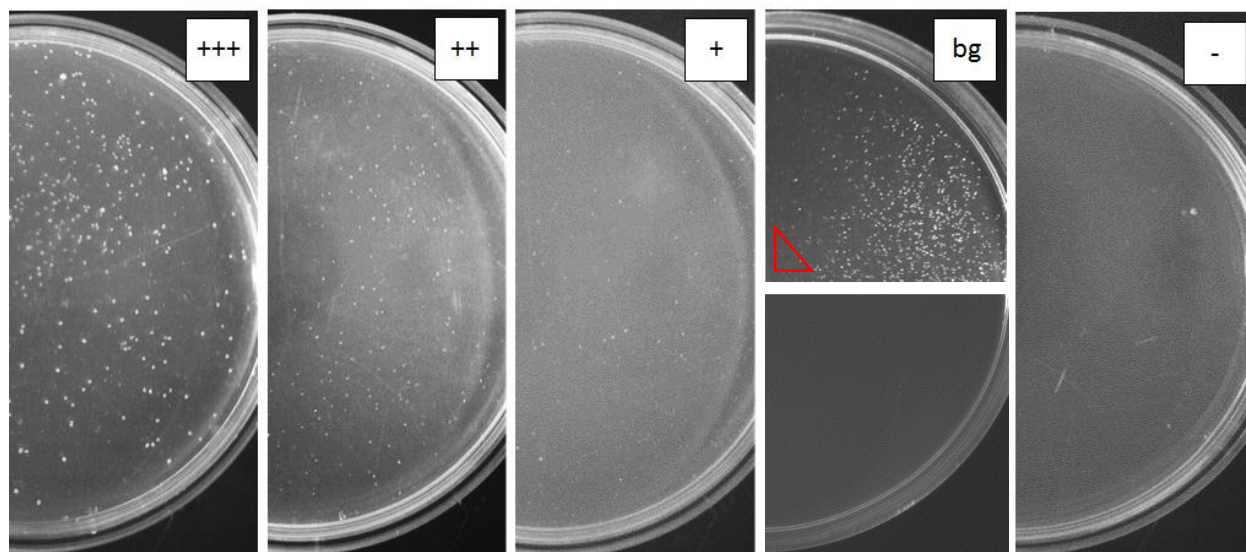
**Table 3.1** Verification that colonies growing on M9/10xcarbon are pseudorevertants

<i>1xcarbon Source</i>	<i>Percent (%) pseudorevertants that grew</i>	<i>Percent (%) pseudorevertants reaching &gt;1 mm in diameter</i>
<i>Glucose</i>	100 (21/21)	66.7 (14/21)
<i>Glycerol</i>	100 (20/20)	85 (17/20)
<i>Pyruvate</i>	86.4 (19/22)	45.5 (10/22)
<i>Succinate</i>	81.3 (13/16)	43.8 (7/16)
<i>Acetate</i>	86.4 (19/22)	18.2 (4/22)

Of the acetate pseudorevertants tested, 86% showed increased levels of growth on acetate compared to the  $\Delta pdxB$  strain but only 18% of the pseudorevertants grew to appreciable size of 1 mm. Pyruvate pseudorevertants grew at similar frequencies to acetate pseudorevertants. However, 45% of the pyruvate pseudorevertants grew well on pyruvate with colonies reaching over 1 mm in diameter. Lastly, succinate pseudorevertants had the highest frequency of colonies that did not grow at the 1x concentration (19%). Only 44% of the pseudorevertants that grew on 1xsuccinate had colonies consistently grow over 1 mm in diameter (Table 3.1)

*Pseudorevertants are able to grow on other carbon sources*

After having cultured and verified that the pseudorevertants had adapted to growing on one of five carbon sources, we set out to determine whether these pseudorevertants grow when plated on the other four carbon sources. We used average colony diameter to determine levels of growth on the plates (Figure 3.1). M9/carbon plates with additional pyridoxine served as controls. Glucose pseudorevertants were able to grow well on glycerol and showed signs of growth on the other carbon sources including acetate (Table 3.2). These pseudorevertants' ability to grow on certain carbon sources better than others might be a function of the carbon source limiting PLP synthesis.



**Figure 3.1** Classification of pseudorevertant growth (irrespective of the carbon source). Plates with red triangle indicate the plate was imaged at an angle to better refract light and highlight colonies. (+++) p277 on M9/1xglucose. (++) s271 on M9/1xglycerol. (+) p276 on M9/1xpyruvate. (bg) y275 on M9/1xpyruvate (-) s231 on M9/1xpyruvate.

Background colonies were only visible when the plate was tilted thus changing the angle of the incident light. These colonies, while appearing in large numbers, are not large enough to be imaged under the same light conditions as the other plates. Figure 3.1 shows a “bg” plate and a “+” plate that at first seem to show similar levels of growth. However, when viewing the

“bg” plate under the same exact conditions as the “+” plate, no colonies are visible. Therefore, the colonies on the “bg” plate are distinct in the fact that their diameters are smaller than the colonies on the “+” plate.

**Table 3.2** Pseudorevertants from M9/10xcarbon were grown on different carbon sources. Growth was characterized after 9-12 days of incubation at 37 °C. The original carbon source the pseudorevertants arose on was indicated by the letter in front of the strain name. (g)lucose, gl(y)cerol, (p)yruvate, (s)uccinate, and (a)cetate.

Strain	pyridoxine	10xcarbon	1xglucose	1xglycerol	1xpyruvate	1xsuccinate	10xacetate
<i>g273</i>	++	+++	+++	+++	++	+	-
<i>g274</i>	+++	+++	++	+++	-	++	++
<i>g276</i>	++	+++	+++	+++	+++	+	bg
<i>g277</i>	+++	+++	+	++	+	+	+
<i>y272</i>	+++	++	++	++	+	bg	bg
<i>y274</i>	+++	++	+	++	-	+	-
<i>y275</i>	+++	+++	+++	+++	bg	bg	-
<i>p231</i>	+++	+++	++	+	+	bg	-
<i>p232</i>	+++	+++	+++	+	bg	bg	-
<i>p233</i>	+++	+++	+	++	++	+	-
<i>p234</i>	+++	+++	+++	++	+	+	-
<i>p271</i>	+++	+	+	+	++	bg	-
<i>p272</i>	+++	+++	+++	++	+	bg	-
<i>p273</i>	+++	bg	+	++	++	+	-
<i>p276</i>	+++	bg	+	++	+	+	-
<i>p277</i>	+++	+++	+++	+++	+++	++	++
<i>p278</i>	+++	++	++	++	+++	+	-
<i>s231</i>	+++	++	+	+	-	+	-
<i>s232</i>	++	+	+	+	+	bg	-
<i>s271</i>	+++	++	++	+	++	++	-
<i>s272</i>	+++	+	++	++	+	+	+
<i>s273</i>	+	-	+	++	-	+	-
<i>s274</i>	+++	+	+++	++	+	++	+
<i>a272</i>	+++	-	+	+++	bg	+	-

+++; good growth, colonies resemble pyridoxine control (>=1mm)

++; decent growth, colonies are slow to appear and often are small (<=0.5mm)

+; weak growth, colonies are present but often don't grow quickly and are very small (<<0.5mm)

-; no growth

bg; background growth

Pyruvate pseudorevertants had the most surprising growth results. Many of these pseudorevertants were able to grow very well on M9/1xpyruvate, M9/1xglucose, and

M9/1xglycerol, reaching over 1 mm in diameter. The strain p277 was able to grow well on all five of the carbon sources and was the only pyruvate strain to grow on acetate (Table 3.2). Interestingly, there were few consistent trends with pyruvate pseudorevertants with regards to growth on certain carbon sources. Each pyruvate pseudorevertant has a different phenotype than all of the other pyruvate pseudorevertants. One consistent trend was that acetate as a carbon source remained the most difficult carbon source for pseudorevertants to grow on, followed next by succinate.

Succinate pseudorevertants struggled to grow on nearly every carbon source with no real correlation with quality of carbon source nor between strains. More energy-rich carbon sources (glucose and glycerol) did not facilitate increased levels of growth. Many strains even struggled to grow well on M9/10xsuccinate and M9/1xsuccinate plates. Strains that grew well on M9/1xsuccinate grew even better on M9/1xglucose. Glycerol pseudorevertants grew well on glucose and glycerol but very poorly on the remaining three carbon sources. Acetate pseudorevertants did not have a large enough sample size to accurately discern trends.

Some pseudorevertants were not able to grow well on the carbon source on which they originated. Reasons for this could be due to the pseudorevertant being able to grow when restreaked, but fell under the category of not reaching 1 mm in diameter, implying it may be on the brink of viability. Only one strain, a272, was not able to grow on its intended carbon source of acetate potentially due to the same reason that nearly all pseudorevertants have difficulty when grown on acetate.

## Discussion

Questions arose as to whether or not every colony that grew on a plate was a pseudorevertant because there were background colonies on every carbon source and large variations in colony size on M9/glucose plates. It is a possibility that these colonies are not actually pseudorevertants but instead are feeding on any remaining PLP left in the media after the original outgrowth. However, after five washes with PBS, it is very unlikely that there is any residual PLP transferred to the plate. In the case of glucose, satellite colonies might be growing by taking up excreted PLP from neighboring colonies. We tested how well pseudorevertants that arose on M9/10xcarbon could grow at a 1x concentration to determine if the strain was able to synthesize its own PLP. If the strain obtained a mutation and regained its ability to synthesize PLP, it was able to grow separated from other colonies.

After restreaking these “pseudorevertants” onto a fresh M9/1xcarbon plate, a majority of these colonies grew, indicating that they are indeed legitimate pseudorevertants. Nearly 100% of the pseudorevertants when restreaked showed growth. However, as expected, the poor carbon sources showed less robust growth (colonies smaller than 0.5 mm in diameter) as these pseudorevertants might still be struggling to metabolize the carbon source (Table 3.1).

After verifying the validity of pseudorevertants, the next question was whether or not its method for synthesizing PLP was dependent on the carbon source. One way to test this question was by plating the pseudorevertant onto the four carbon sources from which it did not originate. Pseudorevertants that are able to grow across the spectrum of carbon sources have acquired a mutation allowing PLP synthesis that is nonspecific to the carbon source. However, pseudorevertants that are only able to grow on glucose and glycerol (y275) may have



a PLP synthesis route that requires specific metabolites or enzymes only present when grown on these carbon sources.

Growing pseudorevertants on plates supplemented with various different carbon sources did not reveal many trends. Glucose pseudorevertants grew the best on glucose and glycerol but also grew decently on pyruvate and succinate. One reason for this trend is possibly due to specific mutations in the *ΔpdxB* strain that arise on glucose allow for efficient PLP synthesis. Glycerol pseudorevertants grew well only on glucose and glycerol. This observation suggests either the need for either a carbon source that can be quickly metabolized, or that PLP synthesis requires specific metabolites and enzymes present only when grown on glucose or glycerol. Succinate and pyruvate pseudorevertants do not show a clear trend, suggesting these pseudorevertants are adapting in unique ways or are synthesizing PLP via different serendipitous pathways.

Inconsistencies between growth on the various carbon sources at 1x concentration to the growth seen on both M9/carbon with pyridoxine and M9/10xcarbon could be due to the frailness of the pseudorevertants and their propensity to further adapt. Many pseudorevertants do not grow well on minimal media and seem to be easily stressed to the point where they no longer grow. Pseudorevertants that did grow were likely adapting as more generations pass. Due to this reason, the cells that are on the plate might not be exact replicates of the cells that were originally plated. This adaptation could cause discrepancies between growth of a pseudorevertant on the various carbon sources.

The carbon source that the pseudorevertant originally grew on seemed to play an important role in its ability to grow on other carbon sources. The metabolome and the suite of

enzymes available to the bacteria appeared to facilitate different paths that all led to PLP synthesis. Certain paths appeared to increase the growth of the bacteria more than others.

Characterization of growth was the first step in determining to what extent the strains have adapted. Experiments characterizing growth on plates are not infallible. To better distinguish these pseudorevertants and to find out exactly how these strains have adapted, whole-genome sequencing of the pseudorevertants was required. The sequence data allowed us to determine exact mutations present.

## Methods

### Verifying pseudoreversion of the colonies that appear on M9/10xcarbon

One pseudorevertant colony was selected after adapting on a M9/10xcarbon plate and transferred to three fresh plates containing M9/1xcarbon, M9/10xcarbon, or M9/1xcarbon with 10  $\mu$ M pyridoxine, which is converted by the  $\Delta$ pdxB strain into PLP via the salvage pathway.<sup>20</sup> The colony was streaked across half of a plate and incubated at 37 °C for 7-9 days. The streaked pseudorevertant strain was deemed a real pseudorevertant if the streaked plate had consistent colony size and morphology after the incubation period.

### Culturing pseudorevertants on various carbon sources

Freezer stocks of pseudorevertants obtained on M9/10xcarbon plates were streaked onto LB/kanamycin plates. A single colony was inoculated and grown overnight in LB/kanamycin. The culture was diluted 100 fold into fresh LB/kanamycin and washed according to the procedure in Chapter 1 "Obtaining pseudorevertants on solid M9 minimal media." For

this experiment, washed cells were diluted before being spread onto plates containing M9/1xglucose, M9/1xglycerol, M9/1xpyruvate, M9/1xsuccinate, and M9/10xacetate. The positive control was a M9/1xcarbon plate containing the carbon source on which the specific pseudorevertant originated supplemented with 10  $\mu$ M pyridoxine. Additionally, the pseudorevertants were plated on M9/10xcarbon to replicate the exact conditions on which the pseudorevertant arose. Plates were incubated at 37 °C for 8-12 days. Colonies that grew to over 0.5 mm in diameter were considered as being able to grow on that carbon source.

## Chapter 4

### Whole-genome sequencing of pseudorevertants

The original goal of the plating experiments described in Chapter 2 was to determine if different carbon sources facilitated the rise of different serendipitous pathways. Since classifying pseudorevertants into categories based on their growth was inconclusive, sequencing pseudorevertant genomes might provide a clear trend in the mutations that arise on different carbon sources, hinting at adaptation strategies. Sequencing data still did not elucidate any clear trends between pseudorevertants because multiple different mutations might achieve the same end goal of growth on M9/carbon. A larger data set will help determine any trends.

## Results

### Whole-genome sequencing of pseudorevertants reveals genomic mutations

After confirming pseudorevertants are able to grow on M9 without supplemental pyridoxine, the next question is what mutations occurred to facilitate growth. Whole-genome sequencing of selected pseudorevertants should reveal any base pair mutation or genome remodeling that occurred as a result of adapting to a specific carbon source or growth without pyridoxine. Figure 4.2 illustrates examples of mutation calls using *breseq*. Eleven pseudorevertants that arose on M9/10xcarbon were chosen based on their growth on minimal

media supplemented with various carbon sources. The goal was to choose pseudorevertants that likely have unique mutations based on differences between their growth (Table 3.2). Three pseudorevertants were chosen from glucose (g273, g276, g277), five from pyruvate (p231, p232, p233, p234, p277), and three from succinate (s271, s272, s274).

From the eleven genomes, three returned no

mutations at all, two showed potentially non-meaningful mutations, and six showed meaningful mutations. (Table 4.2). All three pseudorevertants without mutations originated on pyruvate. Potentially non-meaningful mutations are synonymous mutations (*proX* P159P mutation in g277) or nonsynonymous mutations that replace one amino acid with another of similar structure (*purL* D423E mutation in p277). While these mutations might have an effect on growth of the pseudorevertants, they are not strong enough candidates to pursue at this point in time.

Of the six pseudorevertants showing meaningful mutations, two were of particular interest. One pseudorevertant, s271, which originated on succinate, had a deletion of one base pair in the *trxA* gene, effectively knocking it out. This gene encodes thioredoxin 1 which is involved in reducing cytoplasmic enzymes<sup>21,22</sup> and in multiple pathways including DNA de novo biosynthesis.<sup>23</sup> The second interesting pseudorevertant, s272, had a 15 base pair deletion in the *ilvGEDA* operon leader peptide, *ilvL*. This mutation disrupts the secondary structure of the *ilvL* mRNA<sup>24</sup> resulting presumably in the constitutive expression of the seven genes in the

**Table 4.1** Average sequence coverage of the pseudorevertant genome

<i>Strain</i>	<i>Bases (10<sup>8</sup>)</i>	<i>coverage</i>
<i>g273</i>	9.96	215x
<i>g276</i>	6.57	142x
<i>g277</i>	10.4	224x
<i>p231</i>	8.41	182x
<i>p232</i>	11.8	255x
<i>p233</i>	12.1	262x
<i>p234</i>	5.53	119x
<i>p277</i>	7.90	171x
<i>s271</i>	4.61	100x
<i>s272</i>	10.2	219x
<i>s274</i>	10.6	229x

**Table 4.2** The mutations of 11 pseudorevertants were called using *breseq*. The  $\Delta pdxB$  strain's mutations were present in all other strains and therefore not annotated in the pseudorevertant strains.

Strain	Gene	Enzyme	Mutation
$\Delta pdxB$ strain	<i>pdxB</i>	erythronate-4-phosphate dehydrogenase	Deletion of entire gene
	<i>flhD/uspC</i>	flagellar class II regulon transcriptional activator, with FlhC/universal stress protein	New junction
g273	<i>coaA</i>	pantothenate kinase	Transposable element inserted between TSS and promoter
	<i>ydjE</i>	putative transporter	Transposable element inserted between TSS and $\sigma 70$ promoter
g276	<i>Dpf</i>	fused 4'-phosphopantothenoylcysteine decarboxylase/phosphopantothenoylcysteine synthetase, FMN-binding	N363Y
	<i>ygbI/ygbJ</i>	DeoR family putative transcriptional regulator/putative dehydrogenase	Single nucleotide polymorphism C $\rightarrow$ T (-138/-58)
	<i>rhsA</i>	Rhs family protein, putative polymorphic toxin	Possible deleted region
g277	<i>proX</i>	glycine betaine transporter subunit	P159P
	<i>rhtC</i>	threonine efflux pump	L170L
p231	-	-	None detected
p232	-	-	None detected
p233	-	-	None detected
p234	<i>fdhE</i>	formate dehydrogenase formation protein	E274K
p277	<i>purL</i>	phosphoribosylformyl-glycineamide synthetase	D423E
s271	<i>trxA</i>	thioredoxin 1	$\Delta 1$ bp (15/330 nt)
s272	<i>ilvL</i>	ilvGEDA operon leader peptide	$\Delta 15$ bp (28-42/99)
	<i>yeaR</i>	DUF1971 family protein, nitrate-inducible	Inserted element
s274	<i>argF/ykgS</i>	ornithine carbamoyltransferase 2, chain F; CP4-6 prophage/pseudogene	$\Delta 1$ bp in intergenic region (-55/-69)
	<i>pitB</i>	phosphate transporter	Inserted element in coding region

operon (*ilvX*, *ilvG\_1*, *ilvG\_2*, *ilvM*, *ilvE*, *ilvD*, *ilvA*). To test this hypothesis, plasmids containing each of the operon genes were electroporated into the  $\Delta pdxB$  strain to mimic over expression of a gene. An empty plasmid was also electroporated into the  $\Delta pdxB$  strain to serve as the negative control. The empty plasmid facilitated significant growth when plated on glucose, which contradicts the results seen in Kim *et al.*<sup>5</sup> Therefore, the growth that was seen of the



## Discussion

Whole-genome sequencing of 11 pseudorevertants revealed numerous different mutations that presumably facilitated growth on M9. The data presented here are filled with myriad unique data sets without clear trends between samples, countering the original goal of characterizing trends between pseudorevertants that arose on the same carbon source. Questions arose as to why many of the pseudorevertants' mutations will not likely produce major changes in the bacterium. One pseudorevertant shows only synonymous mutations, another one only shows a missense mutation to a similarly charged amino acid, and three of the strains do not show any mutations. Even synonymous mutations could have an effect on the pseudorevertant, but the odds are unlikely.

The strains that do not have any mutations are of particular interest. All three strains arose on M9/10xpyruvate and continued to show good growth on M9/10xpyruvate in addition to other carbon sources (Table 3.2). However, no mutations were detected. There are certain limitations to the mutations that *breseq* can call including mutations in repeat regions, inversion of large sections of DNA, and rearrangement in repeated DNA sequences.<sup>25</sup> Complex mutations might be hidden within these strains but are not detectable using *breseq*. Further exploring these sequence data might reveal mutations.

Pseudorevertant strains in which mutations were not identified might have lost their mutation during outgrowth on LB when the gDNA was prepped. If the mutation was highly unfavorable (such as a large amplification) it would only have been selected for on M9 due to the PLP-starved conditions.<sup>26,27</sup> Transferring the cells to LB to grow could result in loss of a mutation as it is no longer retained under selective pressure.



Over-expressing the genes in the *ilvGEDA* operon does not directly mimic the mutation found in s272. The goal of this experiment was to determine whether one of the genes alone was a cause for s272's growth. *ilvG* is split into two gene fragments in the K12 strain resulting in a nonfunctional *ilvG* protein. Without the *ilvG* protein, *ilvM* cannot function properly. The *ilvGM* and *ilvM* vectors were not expected to facilitate growth on minimal media as their protein product is nonfunctional in the  $\Delta pdxB$  strain. Since the results of over-expressing the *ilvGEDA* operon genes were inconclusive due to growth of the negative control, it will be important to repeat the experiment or directly recreate the mutation to verify its ability to facilitate growth on M9 minimal media.

In order to gain more insight into the effect these mutations have on the  $\Delta pdxB$  strain, recreation of the specific mutations would be important. Sequencing more pseudorevertant genomes could potentially show trends in mutations or illuminate repeated mutations that play an important role in survival without supplemental pyridoxine.<sup>28</sup> Another way to determine mutations required for adaptation on M9/carbon is to adapt the  $\Delta pdxB$  strain in liquid M9/carbon and sequence these adapted strains' genomes. Liquid medium provides a different environment for adaptation and might be more suitable for adaptation of the  $\Delta pdxB$  strain in pyruvate, succinate, and acetate. Additionally, liquid conditions will better allow characterization of growth by using OD<sub>600</sub> and will rid background colonies as seen on plates. Any bacteria that grow as poorly as the background colonies will likely be a very small fraction of the entire liquid culture. Therefore, upon serial passages, these poorly growing bacteria will be removed from the population, resulting in a more homogenous culture more suitable for characterization.

## Methods

### Whole-genome sequencing of pseudorevertants

The pseudorevertant genomes to be sequenced were chosen based on variation in growth as compared to other pseudorevertants. Chosen pseudorevertants grew well across every carbon source (p277) or were unique between pseudorevertants that arose on the same carbon source (g273 and g277) or different carbon sources (g276 and s271) (Table 3.2). The selected pseudorevertants were grown overnight in LB/kanamycin and 1 ml of cells were harvested. Genomic DNA (gDNA) was purified and collected using Invitrogen PureLink Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA). Two elutions of 25  $\mu$ l ensured high DNA yields. DNA yields were determined using Invitrogen Qubit dsDNA BR Assay Kit (Invitrogen, Carlsbad, CA). DNA purity was determined using a Thermo Scientific Nanodrop 2000. To ensure no RNA was present, 50-100 ng purified DNA was run on an agarose gel to look for RNA contamination at approximately 200 base pairs.

gDNA was sent to Dan Snyder at the Cooper lab in Pittsburgh, PA and sequenced using NextSeq 500 and a HiOutput reagent set with 150 x 2 paired end reads. Raw sequences were analyzed using *breseq*.<sup>25</sup> BW25113 served as a reference genome.<sup>29</sup> Reads were trimmed and recalibrated using *bbmap* and *bbduk*<sup>30</sup> to correctly match the reference genome. Without trimming and recalibration, read mapping only successfully mapped approximately 70% of the reads. After trimming and recalibration, 99.9% of reads were mapped at the cost of total number of reads mapped. However, there still was 100-250x coverage for every nucleotide as determined by dividing the total number of bases sequenced by the total number of bases in

the BW2113 genome. Over100x coverage helped to ensure accuracy of mutations calls (Table 4.1).

#### Overexpressing *ilvGEDA* operon genes in the $\Delta pdxB$ strain

To create electrocompetent cells to use for electroporation, the  $\Delta pdxB$  strain was grown overnight in LB/kanamycin. Cells were then inoculated into 5 ml of fresh LB/kanamycin and grown to mid-log (approximately 0.4 OD<sub>600</sub>). One milliliter of cells was harvested by centrifugation (16100 x g for 1 min) and washed five times in 10% ice-cold glycerol. Cell pellets were resuspended in 50  $\mu$ l of 10% glycerol.

Three genes, *ilvA*, *ilvE*, and *ilvM* were overexpressed on the ASKA plasmid pCA24N vector (Kitagawa et al, 2005). *ilvD*, *ilvGM*, and *ilvED* were expressed in a pTrcHisB plasmid. We did not receive a plasmid containing *ilvX*. Therefore, *ilvX* was not explored in this study. To the electrocompetent cells, 10-100 ng of plasmid DNA was added, and the cells were allowed to incubate on ice for 10 minutes. The mixture was transferred to a cold electroporation cuvette and electroporated using a Bio-Rad electroporator (Bio-Rad Laboratories, Hercules, CA). After electroporation, 950  $\mu$ l of Luria Broth was added to the cuvette to serve as a recovery medium. The solution was transferred to a microcentrifuge tube and placed in a 37 °C shaking incubator for 2 hours. Recovered cells electroporated with pTrcHisB were plated on LB with 100  $\mu$ g/ml ampicillin and cells electroporated with pCA24N were plated on LB with 8  $\mu$ g/ml chloramphenicol to select for the uptake of the plasmid. Plates were grown overnight at 37 °C. Glycerol freezer stocks (-70 °C) were created from colonies that contained the plasmid.

The  $\Delta pdxB$  strain containing one of the plasmids was grown in LB/kanamycin overnight and then inoculated into 5 ml of fresh LB/kanamycin and grown to mid-log. One ml of cells was

harvested by centrifugation and washed five times in PBS. A 2  $\mu$ l aliquot was streaked on M9/1xglucose and M9/1xsuccinate. The  $\Delta pdxB$  strain containing an empty pTrcHisB plasmid served as a negative control.

## Chapter 5

### Long-term adaptation of the $\Delta pdxB$ strain

The  $\Delta pdxB$  strain is able to adapt on solid M9 minimal media supplemented with various carbon sources. Adapting the  $\Delta pdxB$  strain in liquid M9 minimal media with various carbon sources allows better characterization of growth as cultures likely became more homogenous as slower growing bacteria would be outcompeted. The  $\Delta pdxB$  strain was able to adapt when inoculated in liquid M9/glucose and M9/glycerol, paralleling the strain's ability to mutate and grow on solid M9/glucose and M9/glycerol. Adaptation on the other three carbon sources of pyruvate, succinate, and acetate still proved to be a challenge for the  $\Delta pdxB$  strain. In fact, there was no adaptation in these carbon sources. Adaptation did not occur even after allowing the  $\Delta pdxB$  strain time to synthesize all of the enzymes and metabolites required for growth on these three carbon sources. D-alanine, an important component of peptidoglycan, is likely at low concentrations in the  $\Delta pdxB$  strain as a result of PLP depletion. Insufficient peptidoglycan may result in weak cell walls. Supplementation of D-alanine did not facilitate adaptation in pyruvate, succinate, or acetate even though D-alanine supplementation greatly increased the frequency with which the  $\Delta pdxB$  strain adapted in glycerol. The strains that did successfully adapt in M9/glycerol obtained many similar mutations, pointing to the importance of these mutations for PLP synthesis.

## Results

### Long-term adaptation experiments produce adapted strains with various phenotypes

We wanted to test whether the  $\Delta pdxB$  strain would grow in liquid M9 supplemented with one of four carbon sources (glycerol, pyruvate, succinate, and acetate) as we already knew the  $\Delta pdxB$  strain can adapt in M9/glucose (Kim and Copley, unpublished). To test this question, the  $\Delta pdxB$  strain was washed and inoculated in M9 supplemented with one of the four carbon sources ranging from 1x – 10x concentrations of the carbon source. These cultures were incubated at 37 °C while constantly shaken for upwards of 30 days. After this 30-day incubation period, no adaptation was seen in the three poorer carbon sources of pyruvate, succinate, and acetate. Interestingly, not all of the glycerol cultures was able to adapt. Cultures inoculated in 5 ml culture tubes supplemented with 5x, 7.5x, and 10x (1.0%, 1.5%, and 2.0% w/v) glycerol were able to adapt. Adaptation was not seen in M9/1xglycerol. However, an adapted strain that initially grew on M9/10xglycerol was serially passaged into lower glycerol concentrations and eventually was able to grow in M9/1xglycerol (Figure 5.1). This strain grew slowly throughout the entire adaptation experiment, never growing faster than one generation per day.

When the  $\Delta pdxB$  strain was inoculated into culture flasks containing 25 ml of M9/glycerol, adaptation was seen at 2x, 5x, 7.5x, and 10x concentrations. However, only one culture out of 29 was able to adapt in M9/2xglycerol. This culture, m1-2, was passaged for 92 generations (Figure 5.2B). The low frequency of adaptation in M9/2xglycerol may be a result of the strain struggling to synthesize the required enzymes for growth in glycerol or due to lysis of the bacterium as a direct result of insufficient D-alanine, an important component of cell walls.

The concentration of D-alanine would likely be lower in cells lacking PLP because the protein that racemizes L-alanine to D-alanine requires PLP as a cofactor.<sup>31</sup>

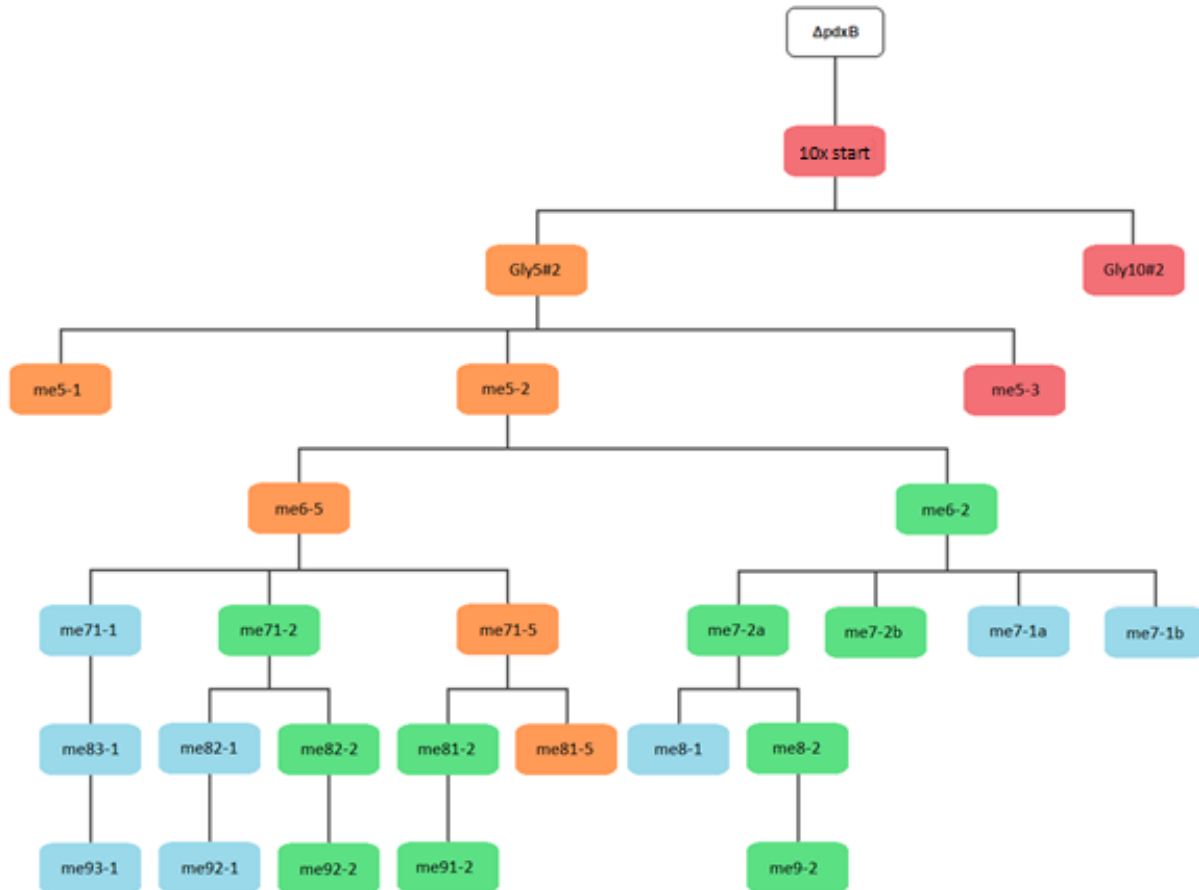


Figure 5.1 Adaptation of the  $\Delta pdxB$  strain in M9 with decreasing concentrations of glycerol. Glycerol concentrations: Red, 10x; Orange, 5x; Green, 2x; Blue, 1x. Growth never exceeded 1 generation per day.

#### Long-term adaptation after preconditioning the $\Delta pdxB$ strain

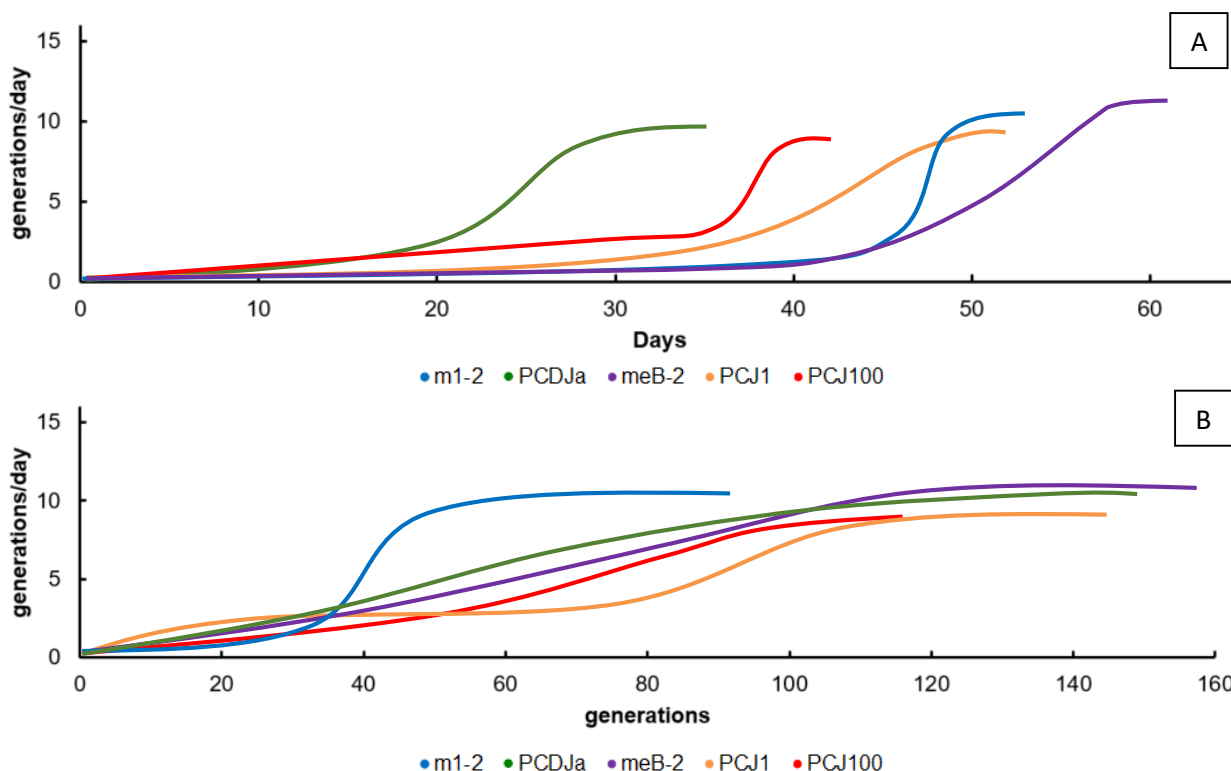
After being transferred from LB to M9/carbon, wild-type *E. coli* experiences nutrient deprivation resulting in a lag time. The lag time indicates the time required for the bacterium to synthesize all of the enzymes and metabolites specific to growth on that carbon source. We see different lag times depending the carbon source available (Figure 2.2). The  $\Delta pdxB$  strain was grown M9/carbon with pyridoxine as opposed to LB in order to reduce the lag time by allowing

the strain to synthesize all of the required enzymes and metabolites. Therefore, when the strain was transferred to M9/carbon, PLP depletion was the only major limitation to overcome. The *ΔpdxB* strain did not have to spend energy and resources synthesizing metabolites that may have hindered its ability to adapt. Preconditioning is this process of growing in M9/carbon with pyridoxine before transfer to M9/carbon.

After the *ΔpdxB* strain was preconditioned, it was given the opportunity to adapt in M9/carbon. Cultures contained concentrations of a carbon source ranging from 1x – 10x. None of the preconditioned cultures containing pyruvate, succinate, or acetate at any concentration of carbon source adapted. Glucose and glycerol cultures successfully adapted at various concentrations including 1x, 2x, and 5x.

However, frequency of adaptation in M9/glycerol was not 100%. The total number of cultures that were able to grow in 25 ml of M9/2xglycerol without preconditioning was only 1 out of 29 cultures. Adapted strains were obtained at greater frequency when adapted in culture tubes and after preconditioning. Two of the strains that were preconditioned and adapted on glycerol, PCJ100 that adapted at 1x glycerol and PCJ1 that adapted at 2x glycerol concentration, were passaged for 113 and 138 generations respectively, and their growth rates were characterized (Figure 5.2). The strains grew very slowly in its first six to eight passages. After passing bacteria to fresh media multiple times, the strain grew at an increasing number of generations per day. Eventually, the strains' generations per day plateaus, as it is no longer gaining large fitness increases.





**Figure 5.2** Five adapted strains were adapted for over 100 generations. The graphs show mock lines of best fit to clearly show the change the generations per day as a function of (A) days (B) generations.

### Long-term adaptation with supplementation of D-alanine

The  $\Delta pdxB$  strain might not adapt frequently in liquid M9 due to a fragile cell wall. Vigorous shaking may prematurely lyse the  $\Delta pdxB$  strain before it has time to adapt. We postulate that the  $\Delta pdxB$  strain has a weak cell wall due to low levels of PLP and therefore low activity of PLP-dependent enzymes. Some PLP-dependent enzymes racemize amino acids, such as the conversion of L-alanine to D-alanine.<sup>31</sup> Low activity of this racemase will lower D-alanine levels, an important component of the cell wall building-block peptidoglycan.<sup>10</sup> Without sufficient peptidoglycan, cell walls may not form correctly and bacteria would be more fragile.<sup>32</sup> By supplementing D-alanine immediately after washing away residual PLP, we aimed to alleviate the lack of PLP and the  $\Delta pdxB$  strain's weak cell wall. D-alanine is not incorporated into

PLP in wild-type *E. coli*,<sup>33</sup> therefore it likely is only aiding in cell wall synthesis and not PLP production.

Data from these experiments showed that D-alanine supplementation at 2 mM in M9/glycerol and M9/glucose greatly increased the number of cultures that adapt in PLP starved conditions. D-alanine supplementation did not facilitate adaptation on pyruvate, succinate, or acetate at any concentration of carbon source. Glycerol cultures that adapted in M9/10xglycerol/D-alanine were then passaged into M9/1xglycerol or M9/2xglycerol and continued to be passaged for over 100 generations. The meB-2 culture adapted (as measured by total number of days to reach adaptation plateau) at the slowest rate among all adapted strains, being slower than the rate of a pre-conditioned culture alone (Figure 5.2A).

*Combining preconditioning and D-alanine supplementation results in increased adaptation in M9/2xglycerol*

If both preconditioning and D-alanine supplementation improve the chances for adaptation in M9/glycerol, combining both techniques should give the best opportunity for adaptation across all five carbon sources. After preconditioning, the  $\Delta pdxB$  strain was inoculated into pyruvate, succinate, and acetate, all supplemented with D-alanine. These cultures did not result in adaptation. Inoculation of the  $\Delta pdxB$  strain in M9/2xglycerol/D-alanine after preconditioning did allow the  $\Delta pdxB$  strain to adapt. One of these adapted cultures, PCDJa, was cultured for 147 generations in M9/2xglycerol. This culture reached its generation per day plateau in less time as compared to the cultures that were only preconditioning (Figure 5.2A).

**Table 5.1** The mutations of 5 adapted strains were called using *breseq*. The  $\Delta pdxB$  strain's mutations were present in all other strains and therefore not annotated in the adapted strains.

Strain	Gene	Enzyme	Mutation
$\Delta pdxB$ strain	<i>pdxB</i>	erythronate-4-phosphate dehydrogenase	$\Delta pdxB$ strain
	<i>flhD/uspC</i>	flagellar class II regulon transcriptional activator, with FlhC/universal stress protein	IS30 inserted
m1-2	<i>ybhA</i>	PLP phosphatase	E232*
	<i>relA</i>	(p)ppGpp synthetase I/GTP pyrophosphokinase	V633G
	<i>serA</i>	D-3-phosphoglycerate dehydrogenase	S373P
	<i>pgi</i>	Glucosephosphate isomerase	+T (1632/1650)
PCJ100	<i>ybhA</i>	PLP phosphatase	E232*
	<i>relA</i>	(p)ppGpp synthetase I/GTP pyrophosphokinase	V633G
	<i>serA</i>	D-3-phosphoglycerate dehydrogenase	S373P
	<i>pgi</i>	Glucosephosphate isomerase	+T (1632/1650)
PCJ1	<i>ybhA</i>	PLP phosphatase	E232*
	<i>relA</i>	(p)ppGpp synthetase I/GTP pyrophosphokinase	V633G
	<i>serA</i>	D-3-phosphoglycerate dehydrogenase	S373P
	<i>pgi</i>	Glucosephosphate isomerase	+T (1632/1650)
meB-2	<i>serA</i>	D-3-phosphoglycerate dehydrogenase	Q301H
	<i>coaA</i>	Pantothenate kinase	A297T
	<i>rpoC</i>	RNA polymerase, beta prime subunit	R337C
	<i>chpB</i>	Toxin of the ChpB-ChpS toxin-antitoxin system	$\Delta 49$ bp (257-304/351)
PCDJa	<i>ybhA</i>	PLP phosphatase	N198K
	<i>ybhJ</i>	Putative hydratase	V498I
	<i>icd</i>	e14 prophase, isocitrate dehydrogenase, specific for NADP+	H366H
			T370T
			L375L
	<i>serA</i>	D-3-phosphoglycerate dehydrogenase	S373P
	<i>rpsQ</i>	30S ribosomal subunit protein S17	$\Delta 1$ bp (233/255)
	<i>livK/panM</i>	Leucine transporter subunit / PanD autocleavage accelerator, pantothenate synthesis	IS5 insertion (-322/-99)
25 genes	<i>icd</i> , <i>ymfD</i> , <i>ymfE</i> , <i>lit</i> , <i>intE</i> , <i>xisE</i> , <i>ymfI</i> , <i>ymfJ</i> , <i>cohE</i> , <i>croE</i> , <i>ymfL</i> , <i>ymfM</i> , <i>oweE</i> , <i>aaaE</i> , <i>ymfR</i> , <i>beeE</i> , <i>jayE</i> , <i>ymfQ</i> , <i>stfP</i> , <i>tfaP</i> , <i>tfaE</i> , <i>stfE</i> , <i>pinE</i> , <i>mcrA</i> , [ <i>icdC</i>	Deletion of genes	

### Genome sequencing of adapted strains reveal multiple similar mutations

The five adapted strains' genomes were sequenced and analyzed using *breseq* (Table 5.1). Three of the strains, m1-2, PCJ110, and PCJ1 had the exact same mutations. All of the

strains except for meB-2 had a mutation in *ybhA*, which hydrolyzes PLP into pyridoxal and inorganic phosphate. The gene that had the most prevalent mutations was *serA*. Mutations were either a nonsynonymous mutation in the enzyme active site (Q301H) or in the enzyme's regulatory region (S373P). In PCDJa, there was missing coverage of 25 genes, indicating a deletion of this region of DNA that corresponds to 15,148 base pairs.

## Discussion

One of the more challenging aspects of adapting bacteria on solid media is modeling their growth. When looking at a plate, the difference between 0.1 mm and 0.5 mm is minimal. In addition, it is difficult to determine whether or not the growing colony is an actual pseudorevertant or a colony growing for a different reason, as we know the  $\Delta pdxB$  strain is right on the brink of survival, evidenced by its ability to grow at 30 °C.<sup>34</sup> For these reasons, it would be beneficial to adapt the  $\Delta pdxB$  strain in liquid media to clearly determine variances in growth via OD<sub>600</sub>. Liquid media also gives opportunity to adapt a single cell lineage for 100 or more generations, providing ample time for the strain to acquire multiple mutations. These mutations are hypothesized to help the  $\Delta pdxB$  strain bypass the block in the synthesis of PLP. The mutations that appear in liquid may be similar to mutations that appear on plates, or they might be completely different.

Interestingly, the  $\Delta pdxB$  strain can only adapt in M9 liquid containing glucose or glycerol while it can mutate and grow on M9 plates containing all five carbon sources. Inoculating the  $\Delta pdxB$  strain in the same carbon concentrations as found on plates did not allow adaptation at 1xglycerol or 2xglycerol. This difference could be due to liquid and solid media environments

being very different from one another. Bacteria grown on solid media benefit from few external forces as the plates are not shaken and cells rest atop of the agar. Conversely, bacteria grown in liquid are shaken constantly to ensure ample oxygenation of the liquid media. Due to this shaking, cells are more susceptible to lysis, especially if their cell walls are not fully intact. The *ΔpdxB* strain likely has a weak cell wall due to a D-alanine deficiency, a direct result of its PLP deficiency. A weak cell wall could lead to the strain's inability to grow in liquid when supplemented with a majority of the different carbon sources and concentrations. The data show that the addition of D-alanine facilitates the *ΔpdxB* strain's adaptation on M9/2xglycerol when it cannot grow on M9/2xglycerol alone.

It was hypothesized that growing the *ΔpdxB* strain in LB and transferring it to M9/glycerol might be strenuous on the bacteria. Factoring in the additional limitation of M9 being devoid of PLP, it is plausible that these conditions combined to become too much for the *ΔpdxB* strain to overcome. The transcriptome of the *ΔpdxB* strain is likely very different on LB as compared to M9/carbon.<sup>13,35-37</sup> By preconditioning the *ΔpdxB* strain in M9/carbon with pyridoxine before transferring the bacteria to the PLP-depleted environment of M9/carbon, the *ΔpdxB* strain can synthesize the required proteins and metabolites necessary to grow on that carbon source. When PLP is removed, the only factor the *ΔpdxB* strain needs to overcome is the PLP depletion and will not require reformatting its transcriptome and proteome. PCJ1 and PCJ100 showed the ability to adapt in M9/2xglycerol (Figure 5.2B) when the *ΔpdxB* strain was incapable of adapting in M9/2xglycerol without first adapting in M9/10xglycerol (Figure 5.1), giving validity to this hypothesis.

Sequence data of adapted strains show for the first time a trend between various strains. Three of the strains have acquired the exact same mutations, while all five of the strains

acquired a mutation in *serA*. Since m1-2, PCJ100, and PCJ1 all have the same mutations, it is probable that there was cross-contamination between the gDNA samples. However, one of these strains still acquired all four of the mutations listed (Table 5.1). We hypothesize that the effect of preconditioning likely only plays a role in the ease with which the strain can first adapt and not in the mutations the strain obtains. There does, however, seem to be a difference between strains that were grown in the presence of D-alanine. meB-2 and PCDJa, both supplemented with D-alanine, have unique mutations. The deletion of a 15kbp region of DNA in PCDJa likely has many different effects on the strain. How these mutations specifically affect PLP synthesis is not yet understood. However, studying mutations in *serA* might prove to be the most interesting as an intermediate in serine synthesis is involved in one PLP serendipitous pathway (Figure 1.2).

The  $\Delta pdxB$  strain adapts in M9 upon supplementing a good carbon source, adding D-alanine, or after preconditioning. All of these strategies have the same goal: to provide the  $\Delta pdxB$  strain enough time to acquire beneficial mutations. After the first mutation helps the  $\Delta pdxB$  strain reach the minimum threshold for growth, subsequent generations continue to adapt at increasing rates until they reach an adaptation plateau at around 100 generations of growth, after which beneficial mutations will come at a decreased frequency.<sup>38</sup> Further exploring the mutations these strains acquired will help determine the effect they have on PLP synthesis.

## Methods

### Long-term adaptation of the $\Delta pdxB$ strain

A single colony of the  $\Delta pdxB$  strain was inoculated into LB/kanamycin or M9/2xcarbon with 10  $\mu$ M pyridoxine (for preconditioning experiments) and grown overnight. Subsequently, a 1:100 dilution of the culture was inoculated into fresh LB/kanamycin or M9/2xcarbon with pyridoxine and grown to mid-log phase. One ml of cells was harvested and washed five times in 500  $\mu$ l ice-cold PBS. The final OD of the washed cells was determined via spectroscopy. Fifteen ml culture tubes containing 5 ml of M9 salts or 125 ml culture flasks containing 25 ml of M9 containing between 1x and 10x M9/carbon were inoculated at a starting OD<sub>600</sub> of 0.001. Cultures were grown in culture tubes unless specified as having been grown in culture flasks. Inoculated culture tubes or flasks were placed in shaking incubators at 37 °C. Once growth of the culture reached greater than 0.1 OD<sub>600</sub>, an aliquot of cells was inoculated into a fresh M9/carbon tube or flask at 0.001 OD<sub>600</sub>. Subsequent passages followed the same protocol. Growth rates were calculated in generations per day:

$$\frac{\text{generations}}{\text{day}} = \frac{\log_2\left(\frac{\text{final OD}_{600}}{\text{initial OD}_{600}}\right)}{\text{days}}$$

Cell lineages were passaged over 100 generations until the generations per day plateaued. After each passage, cells were harvested by centrifugation, washed one time in deionized H<sub>2</sub>O, and stored as both a cell pellet at -20 °C and in M9 15% glycerol at -70 °C.

### Long-term adaptation after preconditioning and with D-alanine supplementation

To alleviate the stress of the transfer from LB to M9/carbon, bacteria were preconditioned on M9/2xcarbon supplemented with 10  $\mu$ M pyridoxine and grown overnight in

culture tubes. Cells were then diluted, grown, washed, and inoculated as describe above into fresh culture tubes or flasks containing M9/carbon (without PLP) and placed in shaking incubators at 37 °C.

To test the effect of D-alanine, the  $\Delta pdxB$  strain was grown and washed as described above and inoculated into culture tubes containing 5 ml of M9/carbon with the addition of 2 mM D-alanine. Only the first culture contained D-alanine. After growth was seen, subsequent cultures contained no additional D-alanine.

Cultures inoculated with washed  $\Delta pdxB$  strain bacteria were all given upwards of 30 days of incubation. If there was no change in OD<sub>600</sub> after this extended incubation period, the cultures were determined as not growing.

#### Whole-genome sequencing of adapted strains

The genomes of five adapted strains (m1-2, PCJ100, PCJ1, meB-2, PCDJa) were sequenced and analyzed following the protocol as described in Chapter 3's methods "whole genome sequencing of pseudorevertants."



## Conclusion

In this honors thesis, I showed that the  $\Delta pdxB$  strain acquires mutations and then is able to bypass a disruption in the PLP synthesis pathway. The  $\Delta pdxB$  strain requires sufficient time and energy to mutate. Carbon sources that are metabolized in glycolysis, such as glucose and glycerol, as well as high concentrations of the other carbon sources, pyruvate, succinate, and acetate, provide the resources required for the  $\Delta pdxB$  strain to adapt on solid medium. When attempting to adapt the  $\Delta pdxB$  strain in liquid, preconditioning and D-alanine facilitate adaptation in glucose and glycerol but not in the other three carbon sources, regardless of their concentrations. After sequencing the genomes of pseudorevertants and liquid-adapted strains, only the adapted strains contained mutations that showed any resemblance of a trend. These strains contained mutations in PLP phosphatase (*ybhA*) and D-3-phosphoglycerate dehydrogenase (*serA*).

The mutation in *serA* is not an isolated incident. In fact, similar mutations were seen by Dr. Juhan Kim, another member of the Copley lab, when he adapted the  $\Delta pdxB$  strain in liquid M9/glucose. The mutations in the regulatory site might prevent inhibition by serine, allowing increased levels of SerA's product 3PHP, which feeds directly into a serendipitous pathway (Figure 1.2). To test this hypothesis, I will grow the adapted strains in the presence of serine and serine with pyridoxine as the positive control. If the strain does not grow with the addition of serine, then the mutation does not prevent serine from inhibiting SerA and likely does not feed into the serendipitous pathway as describe in Kim *et al.*<sup>5</sup> Further exploration of the *serA* mutation will be required to understand its role in PLP synthesis.

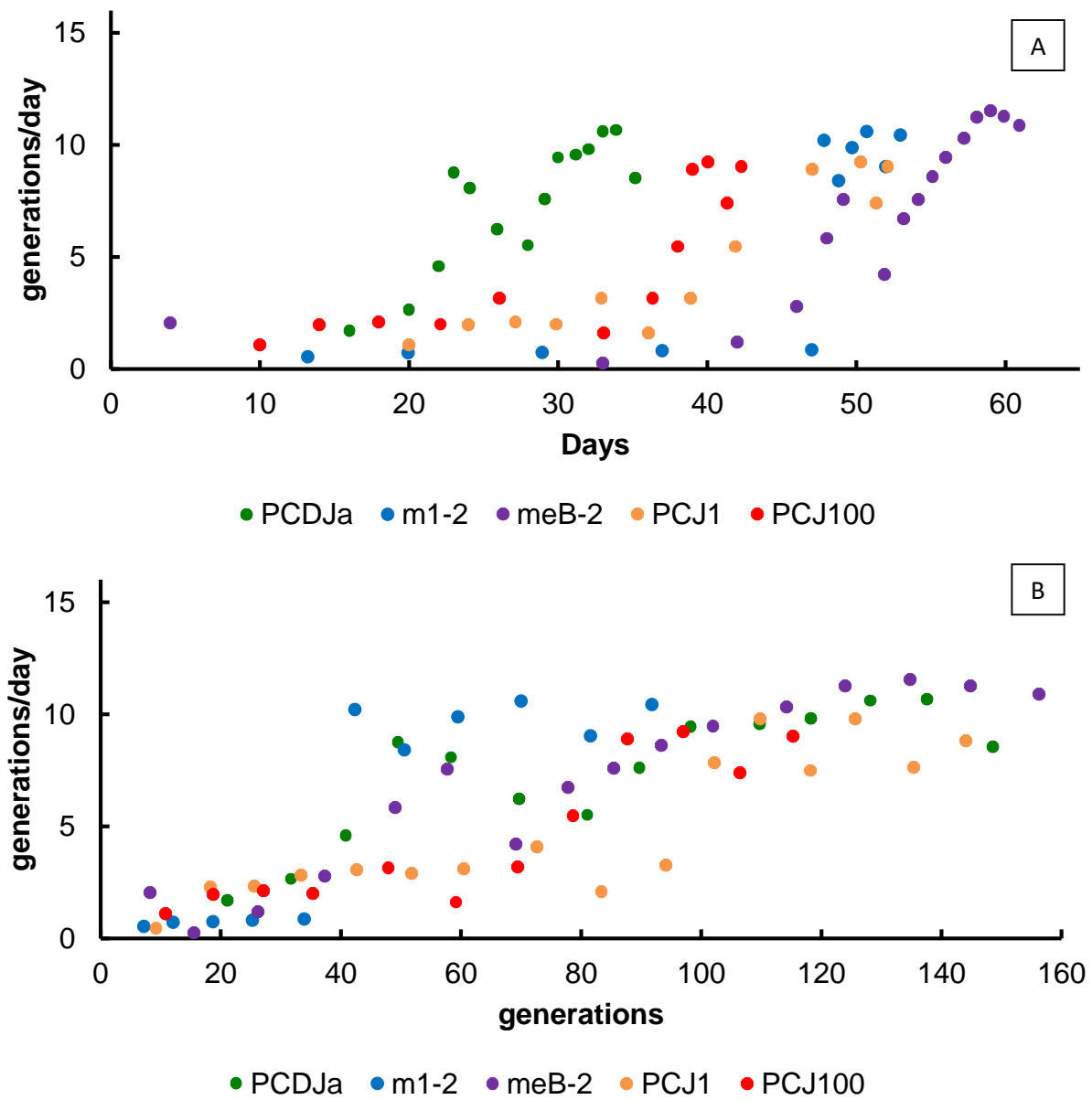
There is a strong correlation between the likelihood of the  $\Delta pdxB$  strain adapting and the environments that allow bacteria with weak cell walls to survive. Increasing carbon concentration, supplementing D-alanine, and growing on solid media all help alleviate the detriment of a weak cell wall. Once the  $\Delta pdxB$  strain adapts, it synthesizes PLP and can racemize its own L-alanine into D-alanine, resulting in a strong cell wall. It is quite remarkable the relative ease with which the  $\Delta pdxB$  strain can adapt and synthesize PLP. In a little over 100 generations, the  $\Delta pdxB$  strain increases its growth rate from less than one generation per day to over ten generations per day, a 900% increase. While the  $\Delta pdxB$  strain is able to adapt and synthesize PLP, it can only do so in limited conditions.

By knocking out *pdxB* and halting PLP synthesis in this *E. coli* strain, we effectively prevent its ability to grow. This simple way of preventing growth of *E. coli* might provide a new target for antibiotic treatments.<sup>39-42</sup> Inhibiting PLP synthesis will prevent *E. coli* from synthesizing their own PLP. The only way they can obtain PLP is from their environment, such as from the human gut. However, humans cannot synthesize their own PLP.<sup>43</sup> Therefore, the concentration of PLP in the human gut may not be at high enough levels to sustain growth of *E. coli*. If there is not sufficient PLP, then the bacteria will not grow. We do see an ability to bypass disruption of this synthesis pathway but only under very favorable conditions which are likely uncommon in the wild. Therefore, any disruption to the PLP synthesis pathway will have an adverse effect on the bacteria.

Further exploring PLP synthesis and the serendipitous pathways that emerge will give a more holistic understanding of the ways in which bacteria are able to adapt to disruption of key metabolic pathways. If we can understand the methods of adaptation, we might be able to better predict how bacteria are bypassing the inhibitory effect of antibiotics or degrading the

inhibitory molecule. Additionally, studying serendipitous pathways may provide insight into the evolution of enzymatic pathways.

## Supplementary Figures



**Figure S1** Cultures of adapted strains from which mock lines were created (Figure 5.2). Strains were serially passaged.

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