

## Adaptive Genetic Mechanisms in *lipA*, a Small Colony Variant of *Escherichia coli*

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### Abstract

Small colony variants (SCVs) are sub-populations of bacteria characterized by their reduced colony size and unique biochemical properties. When *E. coli* mutant strain JW0623 (a SCV referred to as *lipA*) is grown aerobically to log phase it displays resistance to acid (pH3), compared to its parental strain, BW25113 (WT). Using RT-PCR, the expression of genes in log phase cells involved in acid resistance or anaerobic metabolism (*ldhA*, *cfa*, *gadA* and *fnr*) was analyzed in aerobic and microaerophilic conditions to characterize *lipA*. Cyclopropane fatty acid (CFA) lipid analysis was conducted to analyze the percentage of cyclopropane fatty acids in WT and mutant cells grown aerobically and under microaerophilic conditions. Our results demonstrate that mutant cells grown aerobically to log phase exhibit differential expression of select genes and CFA composition resembling WT cells grown under microaerophilic conditions. While the data presented is derived from experiments where the SCV cells are grown to log phase aerobically, there is a notable shift towards anaerobic gene expression. In conclusion, it is evident that *E. coli* SCVs fall under strong selective pressure in hostile environmental conditions and respond with differential gene expression profiles to garner stress resistance.

**Keywords:** SCVs; *lipA*; *Escherichia coli*

### Introduction

Small colony variants (SCVs) constitute a naturally occurring subpopulation of bacteria that differ greatly from their parental counterpart in terms of morphology, growth rate, pathogenicity, and biochemistry [1]. They are often classified as an auxotroph, which are organisms that have lost the ability to manufacture a particular nutrient required for growth and metabolism. Further, SCVs are identifiable by their tiny colony size on rich medium (commonly 1/10<sup>th</sup> the size of the parental strain) often due to auxotrophy for a nutrient required for proper growth [2].

*Staphylococcus aureus* SCVs were first isolated from clinical samples over one hundred years ago. Since then most research has focused on understanding the physical appearance and physiology of *Staphylococcus* SCVs [3]. Over the past twenty years the link between recurring *Staphylococcus* infections and SCVs has been explored to establish a connection between the pathogenesis of disease and SCV phenotypic properties [4].

Due to their morphological and biochemical differences, SCVs present challenges for clinicians who attempt to isolate and identify them from tissue samples. SCVs survive and grow in mammalian cells, and are less susceptible to certain antibiotics than their WT counterparts [1,5]. Moreover, SCVs display resistance to standard antibiotic treatment via biofilm formation leading to disease recurrence [6].

Compared to *S. aureus* SCVs, the literature on *Escherichia coli* SCVs is scarce. Early work [7] reported that *E. coli* SCVs usually appear in populations of bacteria upon exposure to copper stress. Hirsch emphasized that despite Clowes and Rowly's research in 1955 identifying copper as an inducer of SCV formation, the precise relationship between copper and SCV phenotype was unclear.

Hirsch's study of *E. coli* SCVs was significant because he proposed copper as the most likely metabolite to cause profound and lasting physiological and molecular changes in *E. coli*. Further, Hirsch intimated these molecular events include changes in gene expression that contribute to enhanced survival. Hirsch's work suggested copper was an environmental stressor that, in high enough concentration, prompted a switch from planktonic to SCV phenotype in cells over time. SCVs predominate the population of *E. coli* due to the strong selective pressure against planktonic strains from environmental stresses (*i.e.* copper) and due to their resistance to the toxic copper that kills normal cells. Hirsch proposed that the SCV variant was resistant to copper when compared to wild type due to mutation.

*Staphylococcus* SCVs are frequently auxotrophic for three distinct compounds (hemin, menadione, and thymidine). Cytochrome biosynthesis, which requires hemin, is crucial because cytochromes are components of the electron transport chain [8,9]. Menadione is a polycyclic aromatic ketone and a precursor of vitamin K2, which plays an important role in the transport of electrons in respiratory chains. Thymidine is an important component necessary for DNA synthesis [10].

Proctor's work on electron deficient SCVs underpins our understanding of how SCVs achieve long term survival [11]. Proctor showed that deficiency in electron transport or thymidylate synthesis generates the SCV phenotype in *S. aureus*. These electron transport defective SCVs display decreased Krebs cycle activity resulting in reduced electron transport activity overall.

Our work mirrors Proctor's findings pertaining to the current understanding of electron deficient SCVs and persister cells as part of the normal population of bacteria [1]. Accordingly, SCVs appear to arise as a response to harsh environmental conditions including antibiotics, nutrient deprivation and the presence of toxins.

Using *S. aureus* as a model, this study aims to create a molecular and physiological profile of other bacterial SCVs since little work has been published detailing the molecular mechanisms contributing to SCV formation in *E. coli*.

In this study, the SCV *lipA* was generated by insertional mutagenesis and possesses a defunct lipoate synthase, a critical enzyme needed for the insertion of two sulfur atoms to produce a functional lipoic acid cofactor [12].

Lipoic acid, an important sulfur containing organic compound, is needed by microorganisms for proper enzymatic functions. Enzymes that depend upon lipoic acid play a critical role in aerobic metabolism by supplying energy for cellular functions and enhanced survival [13]. Hence, impaired lipoic acid in *lipA* contributes to an energy-deficient state with decreased citric acid cycle activity and overall reduction in electron transport activity. This electron transport chain deficiency disrupts oxidative phosphorylation thereby reducing ATP production.

We propose that this disruption of electron flow is responsible for the SCV characteristics including acid resistance, hydrogen peroxide resistance and slow growth rate (amongst other unique qualities). Small SCV colony size is the result of limited energy for proper growth and metabolism. Hence, impaired lipoic acid synthesis plays a significant role in the SCV phenotypic transformation and contributes to the molecular changes observed in the mutant [14].

In this current study, changes in the expression of genes involved in acid resistance and anaerobic metabolism is compared in the WT and mutant both grown under aerobic condition and low oxygen condition. Using RT-PCR, genes such as *cfa*, *gadA*, *ldhA*, and *fnr* were analyzed to understand global molecular changes that permit *E. coli* SCVs to survive and persist in hostile environments. Whole cell lipid analysis of log phase cells grown under aerobic and low oxygen condition was performed to investigate the role of cyclopropane fatty acids (*e.g.* C17 and C19) play in acid resistance under different oxygen levels.

## Materials and Methods

### Bacterial Strains

The wildtype and mutant SCV strain used in this study are listed on table 1.

Strain	Genotype
BW25113	Parental strain of the <i>lipA</i> mutant*
JW0623	Non-functional <i>lipA</i> gene**

**Table 1:** *E. coli* K-12 strains (descendants of MG1655)

\**E. coli* Genetic Stock Center, Yale University

\*\*Keio Collection from *E. coli* Genetic Stock Center, Yale University

### Media and bacterial culturing techniques

The wild type (WT) strain used in this study is BW25113 and the mutant is JW0623 (*lipA*), a small colony variant created by insertional mutagenesis resulting in a defunct lipoteichoic acid synthase and kanamycin resistance. The *lipA* mutation was verified by DNA sequencing.

Both strains (BW25113 and *lipA*) were cultured aerobically and under conditions of low oxygen in Luria Bertani (LB) or Davis-Mingioli glucose minimal medium to log phase. When needed, lipoic acid was added as a supplement at 5 µg/ml. Incubation of all cultures was performed in a tabletop gyratory incubator at 37°C, with shaking at 150 RPM. Low oxygen condition was maintained by completely filling a screw cap tube to the top with the appropriate medium, limiting the amount of oxygen available. Stock cultures of all strains were kept at 4°C in LB broth, sub-cultured on LB agar plates and maintained at 4°C. Growth of cultures was monitored with a Carolina Spectrophotometer at 580 nm and the growth experiments were run in triplicate.

Microsoft Excel 2013 software was used to generate a growth curve by plotting OD versus time (in minutes) to determine generation time. In all experiments involving LB, WT cells were grown to an OD of 0.60 and the mutant (*lipA*) to 0.30 OD. This is log phase for both strains and CFU determination showed that the number of cells is the same (approximately  $1 \times 10^8$  cells/ml). In all experiments involving minimal medium, WT cells were grown to an OD of 0.60 and mutant cells supplemented with 5 µg/ml lipoic acid were grown to an OD of 0.60.

### Survival assay using inorganic acid (LB, pH 3.0) in LB medium and minimal medium

A survival assay was performed to compare WT and mutant cells incubated in the presence of inorganic acid/pH 3.0. Cells were grown to log phase in both rich and minimal medium using the shaker/incubator at 37°C.

Once log phase in LB medium or minimal medium was reached, the initial CFU was determined by performing an appropriate serial dilution in 0.85% saline and plating 100 µl of the  $10^{-5}$  and  $10^{-6}$  dilutions (in duplicate). The plates were incubated overnight at 37°C, and the colonies were counted the following day to establish a baseline before the strains were subjected to lethal acid shock. The initial number of CFUs was approximately  $1 \times 10^8$  cells/ml.

100 µl of the WT or mutant cells was transferred to 900 µl of Luria Bertani medium/ pH 3.0 or Davis-Mingioli glucose minimal medium/pH 3.0. The cells were incubated for thirty minutes in a 37°C water bath without shaking. Thereafter, the cells were diluted appropriately in 0.85% saline and spread plated on LB agar plates. The plates were incubated overnight at 37°C and the colonies were counted the following day. In all survival experiments, the cells were normalized to  $1 \times 10^8$  CFU/ml. The percent survival = CFU survival/CFU

original x 100. Microsoft Excel 2013 was used to generate a graph showing the percentage survival of both the WT and the *lipA* mutant in rich and minimal medium.

### DNA extraction

For primer verification purposes, genomic DNA was extracted from *lipA* and BW25113 using a Sigma-Aldrich® Bacterial Genomic Miniprep Kit. DNA was eluted with buffer and quantified spectrophotometrically for quality and quantity. All samples had concentrations greater than 200 ng/μl and 260/280 ratios ranging from 1.80 - 2.00.

### RNA extraction

Both the WT and mutant were grown aerobically and under conditions of low oxygen to log phase at 37°C in a rotary/incubator for the purpose of RNA extraction. The protocol from the kit supplied by RiboPure-Bacteria was used to extract RNA.

A digital UV spectrophotometer was used to quantify the RNA and to determine the A260/A280 ratio. After extracting the RNA, the concentration of total RNA was determined to be 800 ng/μl and the A260/A280 ratio was 1.9.

### RT-PCR of select genes in WT and *lipA* mutant (with and without supplemental 5 μg/ml lipoic acid) in aerophilic and microaerophilic conditions at 37°C.

A Qiagen one-step RT-PCR kit was used to perform the reverse transcriptase-polymerase chain reactions. A total of 2 ng/reaction of total RNA was used with a total volume of 25 μl per reaction. Electrophoretic analysis was performed using a 1.0% TBE agarose gel for 30 minutes at 100 volts. New England Bio labs 2-Log DNA ladder was used to estimate the size and intensity of the bands. The housekeeping gene used in this study was 16S rRNA. Table 2 lists gene names and all primers used in this investigation.

Gene	Sequence 5' to 3'
<i>ldhA</i>	for AACCGCTAAAACCTGCCAATG
Lactate dehydrogenase	rev CAATTGCTGCCTGAGAATCA
<i>cfa</i>	for ACCAACTCCCCATCATTTTC
Cyclopropane fatty acid synthase	rev TCATACCACGCCATCAACG
<i>fnr</i>	for ATGATCCCGGAAAAGCGA
Fumarate nitrate reductase	rev TCAGGCAACGTTACGCGT
<i>gadA</i>	for CTGTGACGAAAACACCATCG
Glutamate decarboxylase	rev ACGCAGACGTTACAGAGAGGT
<i>16s rDNA</i>	for TTACCCGCAGAAGAAGCACC
16S ribosomal RNA	rev ACATTTTACAACACGAGCTGAC

**Table 2:** Primers used in experiments.

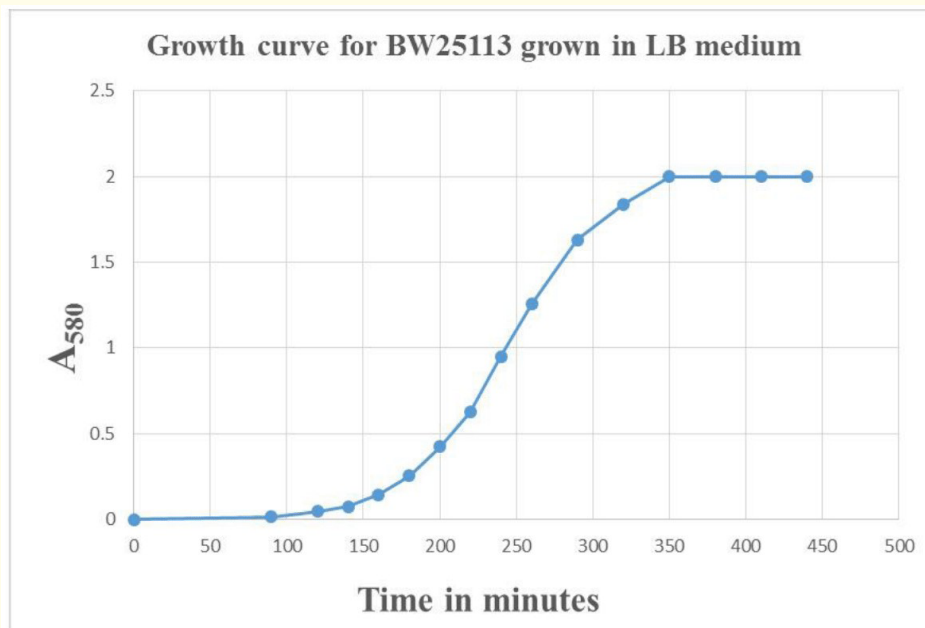
### Cyclopropane fatty acid (CFA) whole cell lipid analysis of log phase grown cells under aerobic and low oxygen conditions

In order to analyze the lipid content of both the WT and *lipA* mutant in log phase, cells were grown as described before with slight modifications [14]. Low oxygen conditions were maintained by completely filling a screw cap tube to the top with the appropriate medium and tightly capping the tubes, limiting the amount of oxygen available. Samples of 10 - 15 mg of were shipped to Microbial I.D. (Newark, DE) for whole cell lipid analysis using the Sherlock gas chromatography system.

## Results

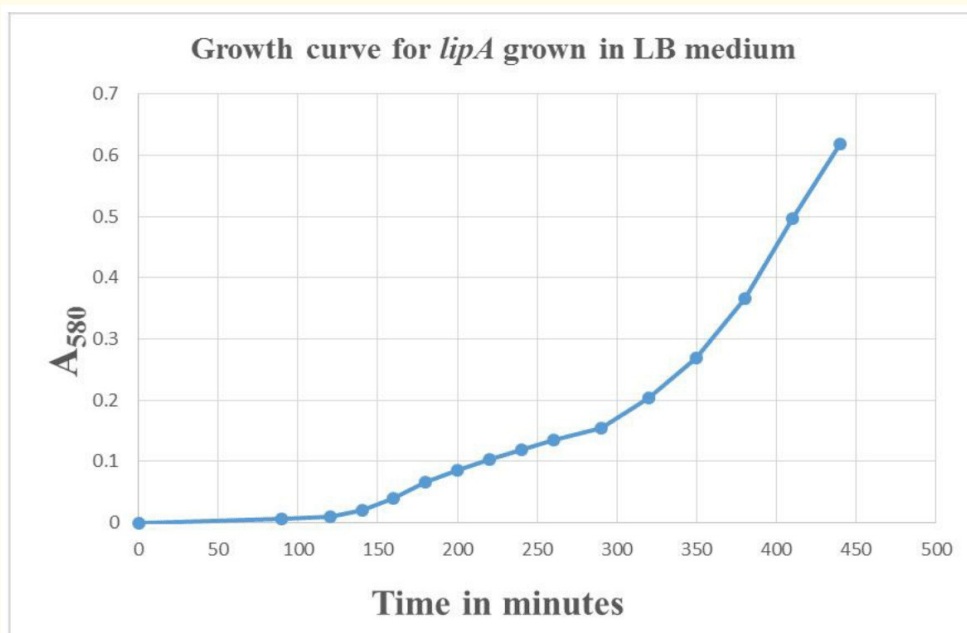
### Growth of WT and *lipA* mutant strain with and without 5 µg/ml lipoic acid in LB medium and Davis-Mingioli glucose minimal medium

When grown in rich medium, the generation time of WT on LB medium was 25 minutes. With the *lipA* mutant, the generation time was 64 minutes in LB alone, but accelerated to 25 minutes when supplemented with 5 µg/ml lipoic acid (Figure 1 and 2). When grown in minimal medium, the mutant failed to grow without lipoic acid supplementation. When grown in minimal medium supplemented with 5 µg/ml lipoic acid, the growth curve of the mutant resembled that of the WT (Figure 3). The SCV *lipA* is auxotrophic for lipoic acid, analogous to results found with *S. aureus* SCVs which lack menadione, hemin, or thymidine.



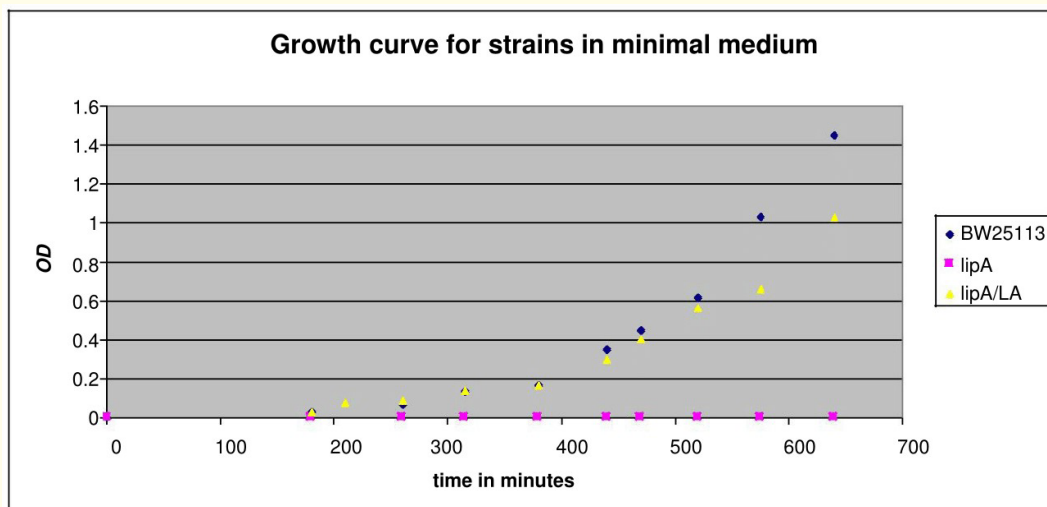
**Figure 1:** Growth curve for BW25113.

Based on the growth curve, the generation time for BW25113 was determined to be 25 minutes based on the average of triplicate experimentation.



**Figure 2: Growth curve for *lipA* cells.**

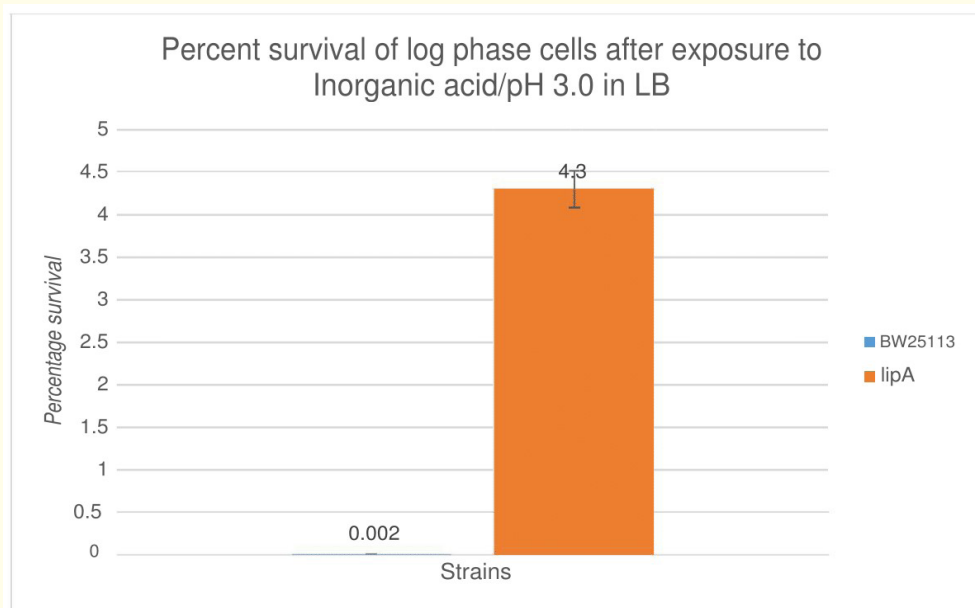
The generation time for *lipA* was determined to be 64 minutes based on the average of triplicate experimentation.



**Figure 3: Growth curve for strains grown in minimal medium.**

**Survival assay using inorganic acid (HCl, pH 3.0) on log phase WT and *lipA* cells grown in rich medium**

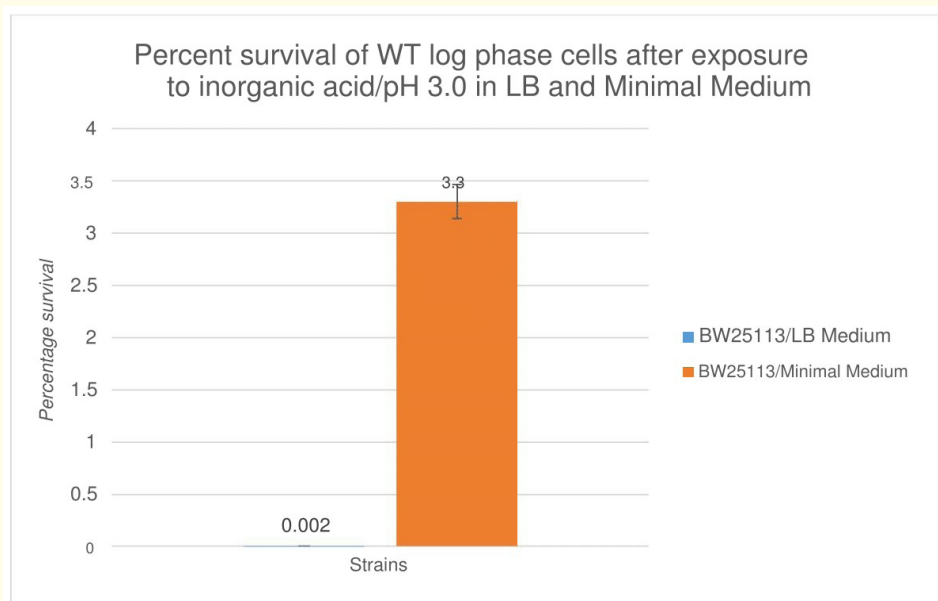
After counting CFUs and normalizing the cell count, it was evident that the *lipA* mutant exhibits enhanced survival compared to WT in the presence of inorganic acid at pH 3.0 (LB, pH 3.0). The survival of the *lipA* mutant was determined to be 4.3% as opposed to 0.002% observed for the WT. On average the mutant exhibits 2,150X better survival than the WT (Figure 4).



**Figure 4:** Percentage survival of log phase cells after exposure to pH 3.0 in LB.

**Survival assay using inorganic acid (HCl, pH 3.0) on log phase WT cells grown in minimal medium**

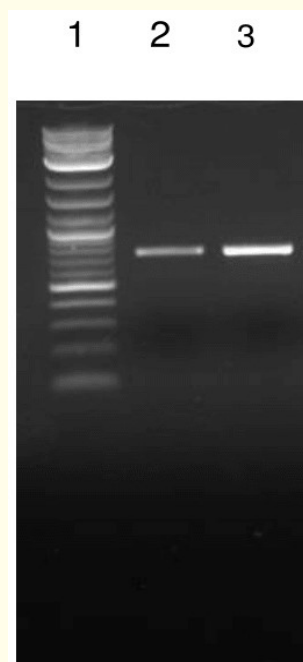
After counting the CFUs and normalizing the cell count, it was evident that WT cells grown in the presence of inorganic acid at pH 3.0 (minimal medium) survived better than when grown in the presence of inorganic acid at pH 3.0 (rich medium). The survival of the WT grown in rich medium was determined to be 0.002% as opposed to 3.3% when grown in minimal medium. On average, the WT grown in minimal medium exhibits 1,650X better survival than the WT grown in rich medium (Figure 5).



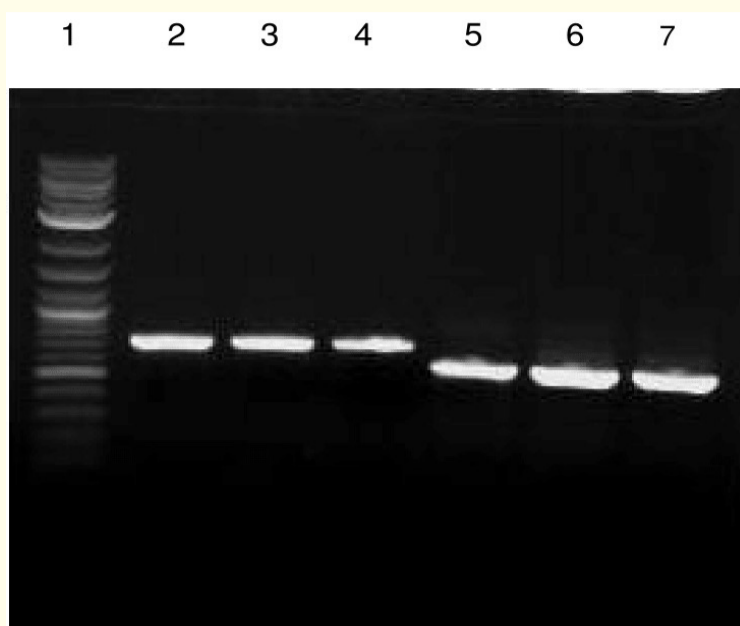
**Figure 5:** Percentage survival of WT log phase cells after exposure to Inorganic acid/pH 3.0 in Rich and Minimal Medium.

**RT-PCR analysis of *gadA*, *cfa*, *ldhA*, and *fnr* in WT, *lipA*, and *lipA* supplemented with 5 µg/ml lipoic acid grown to log phase aerobically or under microaerophilic conditions**

After electrophoretic analysis, the expression of the select genes were found to be elevated in the mutant compared to wildtype when both strains were grown aerobically. However, under conditions of low oxygen, there is a shift in gene expression as both strains displayed similar gene expression. These results indicate that although *lipA* cells were grown aerobically their gene expression was similar to that of a WT strain grown anaerobically or under microaerophilic conditions (Figure 6 to Figure 11).

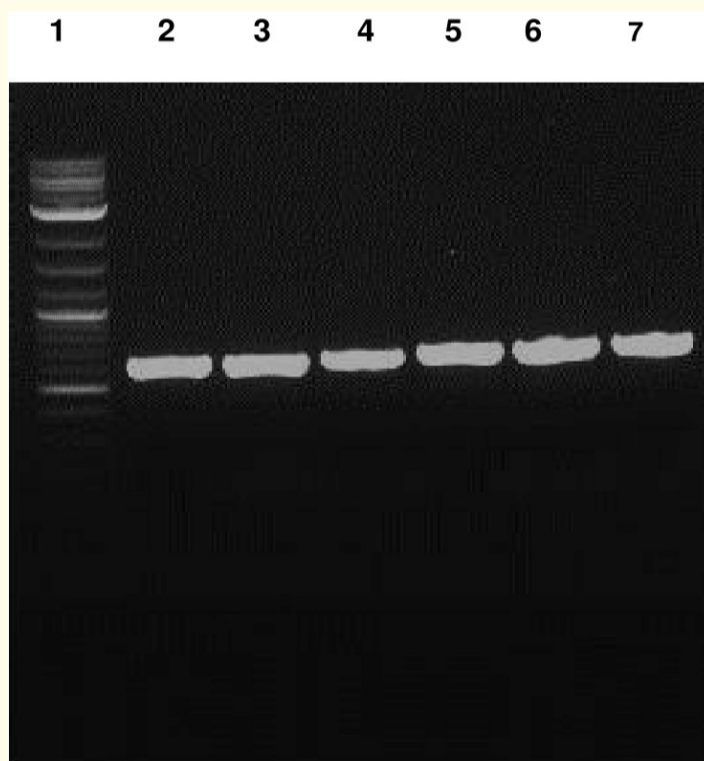


**Figure 6:** RT-PCR analysis of the *cfa* gene in WT and *lipA* log phase cells grown aerobically.  
 Lane 1: Molecular markers  
 Lane 2: BW25113 *cfa* gene  
 Lane 3: *lipA* mutant *cfa* gene



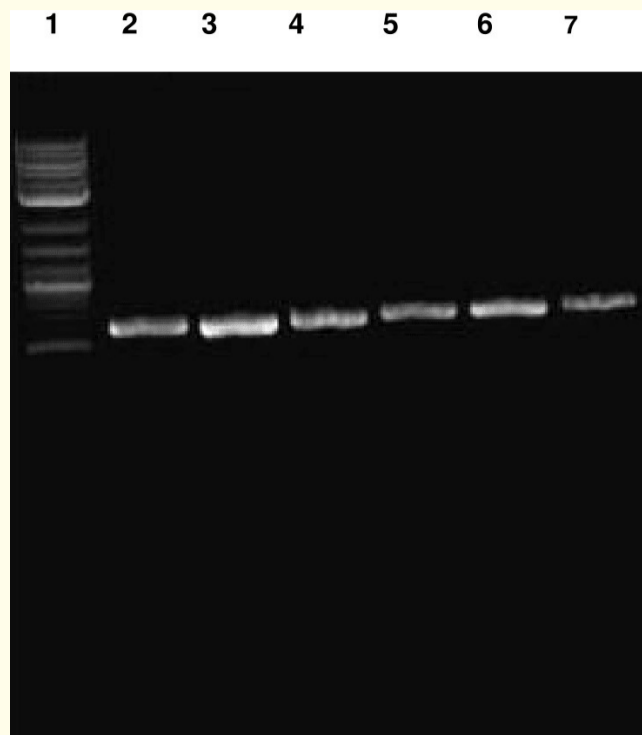
**Figure 7:** RT-PCR of *cfa* and 16S rDNA genes in WT, *lipA*, and *lipA* supplemented with 5 µg/ml grown under microaerophilic condition to log phase.

- Lane 1: Molecular markers
- Lane 2: WT *cfa*
- Lane 3: *lipA cfa*
- Lane 4: *lipA/LA cfa*
- Lane 5: BW25113 16S rDNA
- Lane 6: *lipA* 16S rDNA
- Lane 7: *lipA/LA* 16S rDNA

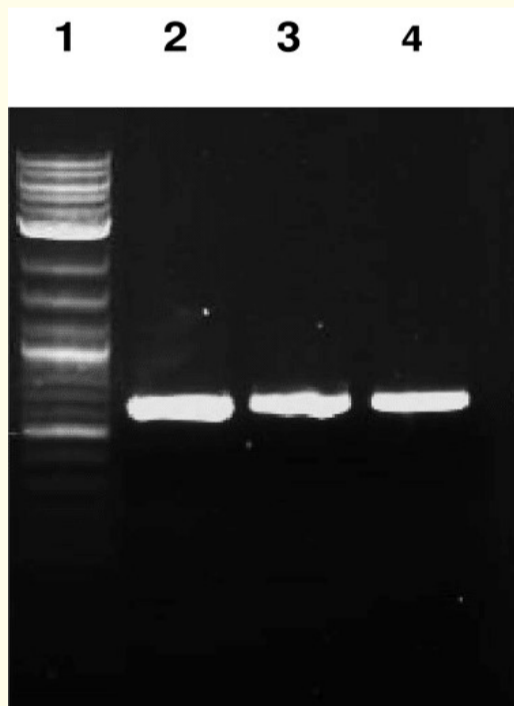


**Figure 8:** RT-PCR of *ldhA* and *fnr* gene in WT, *lipA*, and *lipA* supplemented with 5 µg/ml lipoic acid grown under microaerophilic conditions.

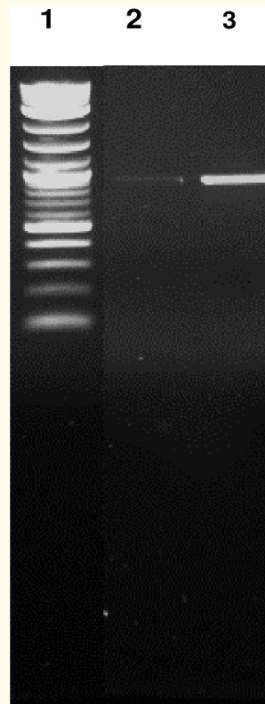
- Lane 1: Molecular markers
- Lane 2: WT *ldhA*
- Lane 3: *lipA ldhA*
- Lane 4: *lipA/LA ldhA*
- Lane 5: WT *fnr*
- Lane 6: *lipA fnr*
- Lane 7: *lipA/LA fnr*



**Figure 9:** RT-PCR of *gadA* and *ldhA* genes in WT, *lipA*, and *lipA* supplemented with 5  $\mu$ g/ml grown aerobically to log phase.  
Lane 1: Molecular markers  
Lane 2: WT *gadA*  
Lane 3: *lipA gadA*  
Lane 4: *lipA/LA gadA*  
Lane 5: WT *ldhA*  
Lane 6: *lipA ldhA*  
Lane 7: *lipA/LA ldhA*



**Figure 10:** RT-PCR of *gadA* gene in WT, *lipA*, and *lipA* supplemented with 5  $\mu$ g/ml grown under microaerophilic conditions.  
Lane 1: Molecular markers  
Lane 2: WT *gadA*  
Lane 3: *lipA gadA*  
Lane 4: *lipA/LA gadA*



**Figure 11:** RT-PCR of *fnr* gene in WT and *lipA* grown aerobically

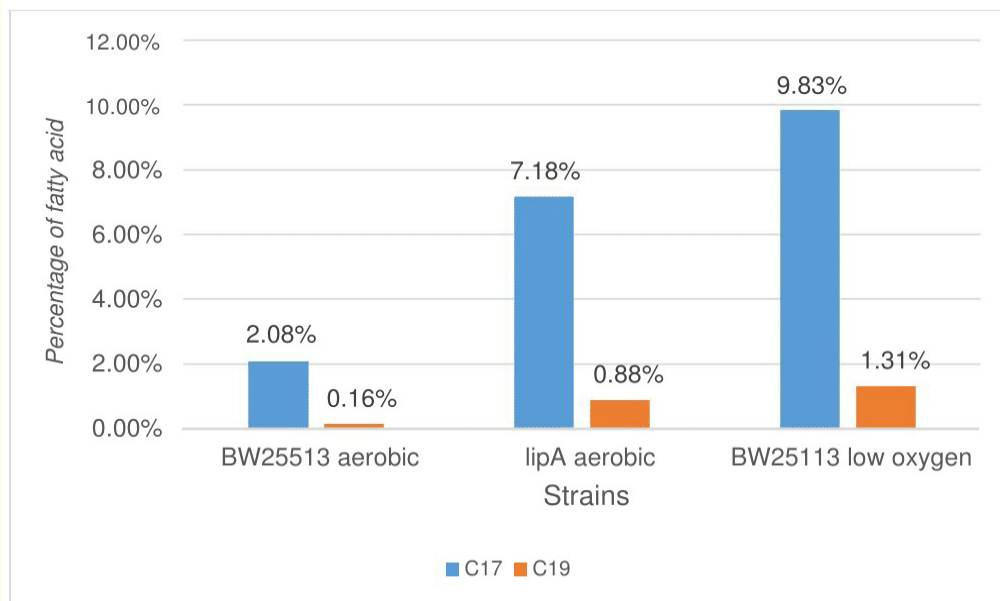
Lane 1: Molecular markers

Lane 2: WT *fnr*

Lane 3: *lipA* *fnr*

### Cyclopropane fatty acid (CFA) whole cell lipid analysis of log phase cells grown under aerobic and microaerophilic conditions

The samples of WT and mutant freeze-dried cells were sent to Microbial I.D. for whole cell lipid analysis. The percentage of C17 and C19 cyclopropane fatty acids were of interest to us. Based on the results, the mutant had a decidedly higher CFA content than wild type in cells grown aerobically to log phase. The C17 CFA content of the SCV was 7.18%, but only 2.08% in the WT. The C19 CFA content was 0.88% and 0.16%, respectively. All experiments were done in triplicate. When WT was grown under low oxygen conditions to log phase, the CFA content was 9.83% for the C17 CFA and 1.31% for the C19 CFA (Figure 12). The CFA levels on the mutant resembles the levels of the WT grown to log phase under microaerophilic conditions.



**Figure 12:** CFA content of WT and *lipA* grown to log phase aerobically and WT grown under microaerophilic conditions.

## Discussion

To briefly summarize the identifiable changes in the SCV *lipA* in this study: slower cell growth, cyclopropane fatty acid composition modulation, important genes for anaerobic growth/metabolism are elevated in the mutant, and growth of the strains in minimal medium appears to alter the survival percentage of the WT strain indicating the expression of certain genes that confer survival is affected by media type.

This is the first study to demonstrate that these changes come from impaired lipoic acid synthesis and can be reversed by supplementation with lipoic acid. Moreover, no published work highlights multiple phenotypic alterations that occur in response to a single known mutation in SCVs.

In previous studies in our laboratory (Paratore, 2015), SCVs grown aerobically display differential gene expression indicative of anaerobic or microaerophilic growth resulting from a global stress-response state within the mutant. This study shows the SCV *lipA* behaves similarly with respect to altered gene expression.

*LipA*'s response is in line with Darwinian fitness: individual fitness is manifested through its phenotype. Since phenotype is influenced by environment as well as by genes, then the benefit of a given phenotype can be different in different environments. Ordinarily, the adaptive burden on a microorganism to simultaneously modify cyclopropane fatty acid composition of the bacterial cell wall and slow growth would be substantial, putting the organism at a disadvantage. However, since the fitness of the genotype is an averaged quantity, it will reflect the reproductive outcomes of all individuals with that particular genotype in a given environment or set of environments [15,16].

As noted, the majority of SCV study has been devoted to work with *Staphylococcus aureus*. Research has shown that certain *S. aureus* SCVs originate by mutations in metabolic genes (e.g. genes needed for proper functioning of the electron transport chain), resulting in the emergence of auxotrophic bacterial subpopulations (Proctor, *et al.* 1995). This study demonstrates that a known mutation in a critical gene (*lipA*) can lead to the SCV phenotype.

More focus is laid on *S. aureus* SCVs than *E. coli* SCVs due to their clinical importance related to their reduced susceptibility to aminoglycoside antibiotics. *S. aureus* SCVs are commonly auxotrophs for hemin, menadione or thymidine, resulting in electron transport chain defects and consequently reduced membrane potential [17].

Our data presented on *fnr* expression promotes certain questions. Why should *fnr*'s expression be so drastically high? Is this a sign SCV *lipA*, although grown aerobically, perceives that it is in anaerobic conditions? *Fnr*'s expression has also been shown to be high in the SCV.

IH9 and SCV IH8 in recent experiments conducted in our laboratory (Paratore, 2015). This may be a prerequisite or requisite for the SCV phenotype in *E. coli* SCVs independently isolated in our laboratory.

From an evolutionary prospective, it is evident that *E. coli* SCV survive better due to gene expression associated with stress resistance (genes including *ldhA*, *cfa*, *gadA* and *fnr*). We present evidence that *E. coli* SCVs exhibit anaerobic gene expression and propose that these variants have an inherent gene program that allows them to survive and continue evolving regardless of environmental stressors. These SCVs have demonstrated the ability to endure sub-optimal environments by triggering survival strategies that will allow them to persist until more favorable conditions emerge.

Most of the work done over the past several decades on *Escherichia coli* SCVs focused on understanding the physiology and morphology of such strains with special attention paid to the unique morphology and physical attributes associated with these variants. Our work, though not complete by far, offers a "molecular view" of these organisms and provides us with a different approach to our understanding of how they evolve and ultimately survive [18,19].

### Conclusion

In conclusion, when the mutant is grown aerobically in LB to log phase, it shows remarkable resistance to acid (pH3), compared to its BW25113 (WT). However, when grown in minimal medium, both the WT and the mutant strains display resistance to acid stress (pH3). In 2013, Riggins (and collaborators) provided evidence that *E. coli* displays different phenotypic properties depending on the type of medium it is propagated in. More importantly, when the SCV is grown aerobically to log phase, the expression of *ldhA*, *cfa*, *gadA* and *fnr* genes resemble the expression of the WT grown anaerobically to log phase. The above data indicates although these SCV cells were grown to log phase aerobically, their gene expression is that of anaerobically grown cells. As a corollary, the growth of the strains in minimal medium appears to alter the survival percentage of the WT strain and might indicate that the expression of certain genes that confer survival is affected by media type. Anaerobic functions are expressed (e.g. *Fnr*) even though the cells are grown aerobically indicating that cells with deficient electron transport could believe they were in an anaerobic environment (and thus demand more oxygen capture by the *Fnr* protein). Our results expand the understanding of the physiological and molecular characteristics of an *Escherichia coli* SCV. Future work includes analyzing more closely the relationship between media type and percentage of surviving cells.

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