Biomarkers to Identify and Characterize Small Colony Variants of Escherichia coli K-12

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Abstract

Small colony variants (SCVs) were initially discovered over a century ago, and were characterized morphologically by their small colony size, and convex, smooth appearance. There has been extensive study of Staphylococcus aureus SCVs because of their association with persistent infections such as skin, soft tissue infections, chronic osteomyelitis, and cystic fibrosis. In contrast to the abundance of studies on staphylococci, especially pathogenic S. aureus, relatively little has been investigated in E. coli SCVs although they have been associated with chronic, recurring infections, such as urinary tract infections, and prosthetic hip infections. Three independently isolated E. coli SCVs were investigated in this study; IH3, from pH 3.0 LB medium, IH9 from commercial apple cider (pH 3.7), which is a weak organic acid, and the mutant strain, JW0623, a lipoic acid (lipA) auxotroph obtained from the Keio collection. Neither IH3 nor IH9 was found to be auxotrophic. The strains were grown to log-phase in neutral LB, followed by RT-PCR and electrophoretic analysis. Five genes were found to be upregulated in all three strains: fecR, wcaC, wcaK, gadA, and cfa. These genes appear to be good candidates for molecular biomarkers that could be used to identify E. coli SCVs from clinical isolates.

Keywords: small colony variants; biomarkers; acid resistance; auxotrophy.

- ***Introduction***

The discovery of small colony variants SCVs) dates to 1910 (1). Since that time, several genera have been associated with the SCV phenotype. These include studies on staphylococcus, predominantly on *S. aureus*, enterococci, *Streptococcus tigurinus* and *Escherichia coli* (1-4). Two of the predominant phenotypes of small colony variants is their small colony size, considered to be 1/10 the size of a normal colony, and auxotrophism, especially for hemin, menadione and thymidine (1, 5, 6). The auxotrophic phenotype is due to genetic mutations, and the deficiency in hemin or menadione will lead to a deficiency in cytochrome biosynthesis, which ultimately results in poor ATP synthesis and slow growth (1, 5).

Whereas there has been a considerable focus on *S. aureus* SCVs, relatively little attention has been paid to *E. coli* SCVs. In the mid - 20th century there were a few publications on *E. coli* SCVs, but these studies focused primarily on their isolation, and their morphological and metabolic properties (7-10).

Recently, a report was published on a novel *E. coli* small colony variant, that.is a lipoic acid auxotroph (5). A deficiency in the ability of *E. coli* to produce lipoic acid is an important cofactor for the pyruvate dehydrogenase complex, and the inactivity of this complex can lead to a decline in electron transport, and consequently a decrease in the synthesis of ATP.

Small colony variants are of interest for their own study, but additionally, and separately, should be studied because they are involved in chronic and recurrent infections. With respect to *E. coli* SCVs, reports have been published on these types of infections (4, 11, 12). An important element in these studies is the difficulty in being able to properly identify the SCVs on the basis of biochemical testing (4, 12). It would be helpful if molecular biomarkers could be found that could be used for the identification of SCVs, in this case *E. coli*.

In this study three small colony variants were employed, each was independently obtained from their respective parent. IH3 was isolated upon incubation of its parent HfrH3000 in pH 3.0 LB medium; IH9 was isolated upon incubation of its parent BW7261 in apple cider (pH 3.7), and strain JC6023, a lipoic acid auxotroph was obtained from the Keio collection.

The genes chosen for this study include *cfa* (cyclopropane fatty acid synthesis), *wcaC* and *wcaK*, (colonic acid synthesis involved in biofilm formation), *fecR*, (iron transport), and *gadA*. These genes were chosen because of their role in stress resistance (32).

- ***Materials and Methods***

2.1 Bacterial strains

The wild types and mutant SCV strains used in this study are listed in Table 1.

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

Strain	Genotype
BW25113	Parental strain of JW0623
JW0623	Non-functional *lipA* gene* - Keio Collection
BW7261**	Parental strain of IH8 and IH9
HfrH3000	Parental strain of IH3*
IH3	SCV isolated by acid resistance from HfrH3000
IH9	SCV isolated by acid resistance from BW7261

Keio Collection from E. coli Genetics Stock Center, Yale University

*Isolated from BW25113

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

2.2 Media and bacterial culturing techniques

The wild type *E. coli* K12 strains used in this study were HfrH3000, BW7261 and BW25113. The mutant strains (SCVs) isolated from them were respectively, IH3, IH9 and JW0623 (see Table 1). All strains were cultured in Luria Bertani (LB) medium to log phase. The incubation of all cultures was done in a New Brunswick Scientific Environmental gyratory shaker at 37°C with shaking at 200 RPM. Stock cultures of the strains were kept at 4°C in LB broth, and sub-cultured also on LB agar plates and maintained at 4°C. For long term storage the bacteria were maintained at -80°C in LB 30% glycerol.

2.3 Screening for acid-resistant mutants.

HfrH3000 was grown to log phase in LB medium, and 100 µl were diluted into 900 µl of pH 2.9 LB, which brought the pH to 3.0. These cells were incubated at 37°C for 1 h with shaking at 200 RPM. Subsequently, 100 µl of this culture was diluted into 10 ml of LB medium and regrown to log phase. This process was repeated for three rounds to enrich for acid-resistant mutants. After round 3, cells were plated on LB agar (neutral pH). Small and large colonies were picked and restreaked with sterile toothpicks onto fresh (neutral pH) LB plates. With the small colonies it was of importance to observe that they were truly small and that they did not revert to large colonies at a high rate. A small colony of interest identified as IH3 was saved. IH9 was isolated from strain BW7261 by using the same strategy, but selecting in apple cider (pH 3.7), which represents a weak organic acid.

2.4 Growth rate analysis of WT and mutant strains

In order to understand the generation time and the growth pattern of the strains, the cells were grown and the optical density was recorded at an absorbance of 580 nm using a Carolina model 65-3303 spectrophotometer. 15.0 ml of sterile LB medium was aseptically transferred to a sterile 125 ml flask and inoculated with 100 µl of log phase bacterial cells and incubated at 37°C with shaking at 200 rpm in a New Brunswick Scientific Environmental gyratory shaker.

A growth curve was generated using Microsoft Office Excel 2019 software after plotting the optical density versus time in minutes. Log phase growth represents approximately 10^8 cells/ml.

2.5 RNA extraction from the WT and mutant strains

Both the WT and the mutant strains were grown at 37℃ in a rotary/incubator to log phase for RNA extraction. The protocol from the kit supplied by RiboPure-Bacteria was used to extract the messenger RNA as described in our previous work (Santos and Hirshfield, 2016). A digital UV spectrophotometer was then used to quantify the RNA and to determine the A260/A280 ratio. After extracting the RNA, the concentration of total RNA extracted was determined to be 600 ng/µl and the A260/A280 was determined to be 1.9. The RNA was aliquoted to Eppendorf tubes to a final concentration of 10ng/µl to use for the RT-PCR reactions.

2.6 RT-PCR procedure to investigate the expression of selected genes

After the concentration of the RNA was established, the protocol from the Qiagen One-Step® RT-PCR kit was followed to perform the reverse transcriptase-polymerase chain reactions. A total of 10ng/reaction of total RNA was used. The total volume of each reaction was 25µl.

2.7 Electrophoretic analysis

Analysis was done by electrophoresis using a 1.0% TBE agarose gel for 30 minutes at 100 volts. The New England Biolabs 2-Log DNA ladder was used to estimate the size and intensity of the bands. The house keeping gene used in this study was 16S rRNA gene. Table 2 lists gene names and all primers used in this investigation.

Table 2. Gene Name and primers used in this investigation.

Gene	**Sequence 5´ to 3´**
wcaC biofilm colanic acid gene	for CGCAGGTCAAACATACG rev AACTGAATACCAGCGCAT CC
wcaK biofilm colonic acid gene	for GTTCTTCCTGGCTGCTCAAC rev TAGCTGTCAATGCCCGTACA
cfa cyclopropane fatty acid synthase	for CTGGATAGTCGGCAAAGAGC rev TGCCAGAGCTGAATATCACG
16s rDNA 16S ribosomal RNA	for TTACCCGCAGAAGC CC rev ACATTTCACAACACGAGCTGAC
fecR Iron transport gene	for GCAACAGTGGTATGAACAGG rev GGTATTTTTCAGCGGGAACG
gadA glutamate decarboxylase	for ACTTTCGGCGTGACCTACAC rev ACGCAGACGTTCAGAGAGGT

2.8 RNA isolation and extraction

The strains were grown to log phase and total RNA was extracted using an Ambion Ribo Pure™ Bacteria Kit (Applied Bioscience). Genomic DNA was eliminated by RNase-free DNase I during the isolation. Each RNA sample was quantified spectrophotometrically for quality and quantity. The RNA was then eluted into elution buffer and stored at -80°C. RNA ranged in concentration from 300 - 900 ng/µl. The 260/280 ratio for all samples ranged from 1.8 – 2.0.

2.9 Cyclopropane fatty acid (CFA) whole cell lipid analysis of log phase cells.

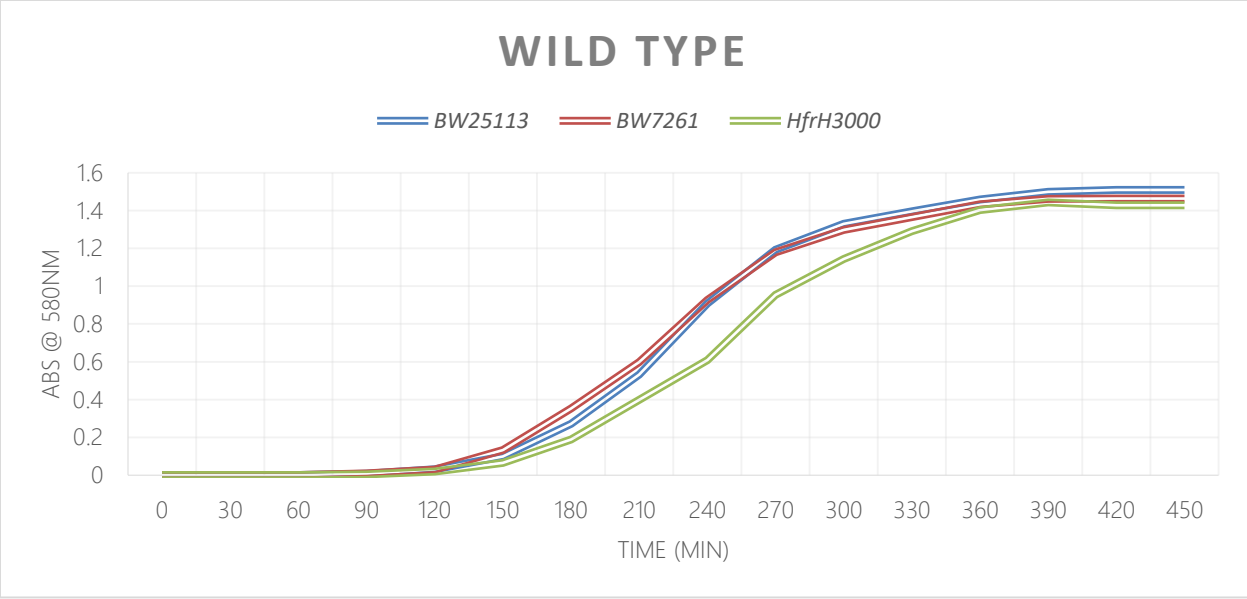
In order to analyze the lipid content of both the WT cells and the SCV mutants in log phase, cells were grown in a shaker/incubator at 37°C. A 100 ml volume of LB medium was used to grow the mutant cells to log phase, and a 50 ml volume was used to grow the WT to log phase. After the desired OD was reached, the cells were poured into 50 ml sterile conical tubes, and centrifuged for ten minutes at 8,000 RPM in a JA-12 rotor at 4°C using the Beckman Coulter centrifuge. After 10 minutes, the supernatant was discarded, and 10 ml of 10mM Tris-HCl buffer (pH 7.4) was added to each tube. The cells were washed twice using this buffer for 10 minutes at 8,000 RPM. After the second wash, the supernatant was discarded, and each strain was then suspended in 5.0 ml of the buffer. The cell suspensions were transferred to a 65 or 75 ml pear-shaped sterile flask and quick frozen in dry ice acetone. They were then stored at -20°C until the following day when they were lyophilized using a Virtis-Lyophilizer, after which the samples were removed and stored at -20°C. Ten to 15 mg of each sample was shipped to Microbial I.D. (Newark, DE) for whole cell lipid analysis using the Sherlock gas chromatography system.

- **Results and Discussion**

3.1 Growth rate analysis of WT and mutant strains.

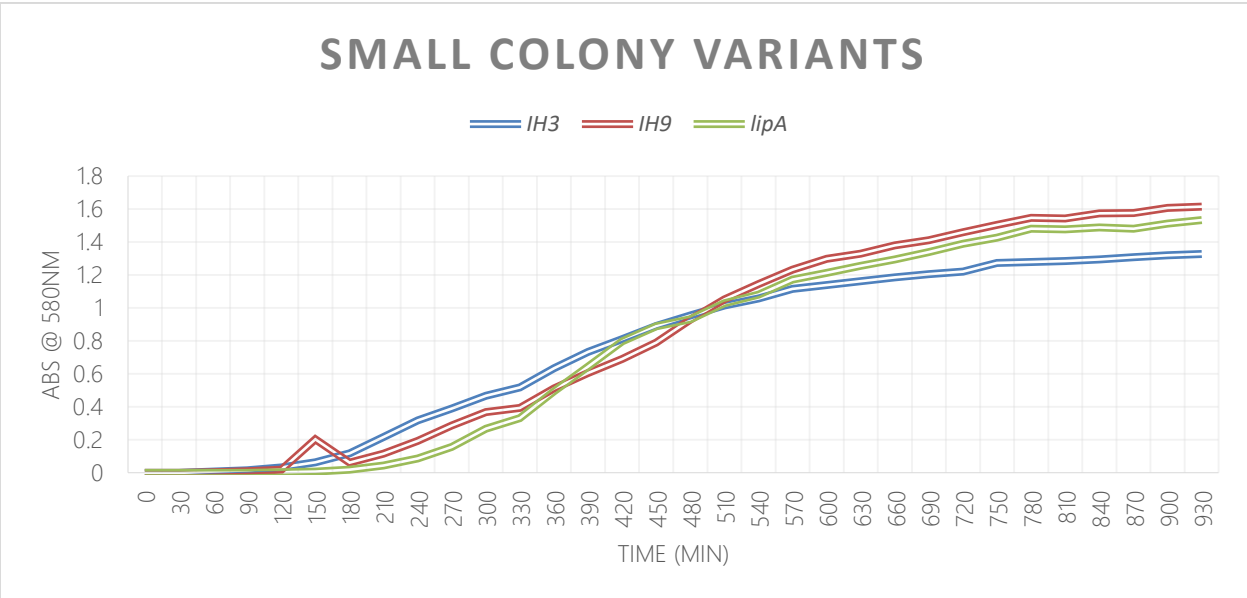
MS Excel 2019 was used to generate a growth curve after gathering the data and plotting optical density versus time (in minutes). The average of three experiments was plotted. Based on the growth curve, the generation times of the WT strains were determined to be approximately 30 minutes in duration and the generation time of the three mutant strains were determined to be approximately 60 minutes in duration (Fig 1 and Fig 2).

Figure 1. Wild Type Growth Curves



Growth curve of wild type strains including BW7261, BW25113, and HfrH3000.
Generation time for BW25113 (30 minutes), generation time for BW7261 (30 minutes), generation time for HfrH3000 (30 minutes).

Figure 2. Mutant Strains Growth Curves



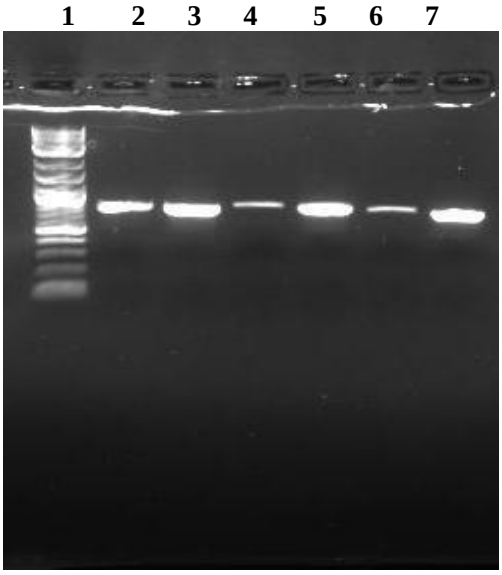
Growth curve of mutant strains including IH3, IH9 and *lipA*. Generation time for IH3 (60 minutes), generation time for IH9 (60 minutes) and generation time for *lipA* (60 minutes).

Based on the growth curves, the OD at which wild type cells were at log phase was determined to be between 0.60 and 0.65 at 580nm. The mutant cells were determined to be at log phase at an OD between 0.30 and 0.35 at 580nm. Any experiment conducted at log phase was done after growing the cells to these OD ranges at 37˚C.

3.2 RT-PCR analysis of selected genes in WT and mutant strains

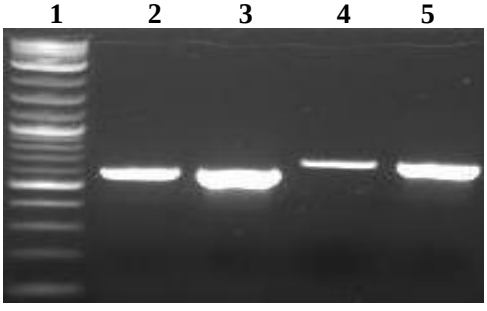
After running the electrophoresis gel and analyzing it with the bio-illuminator, the expression of *cfa, wcaC, wcaK, fecR* and *gadA* genes were found to be elevated in all three mutants as compared to their respective wild-types (Figures 3 to 8). Due to their unusually small size/morphology and unique fastidious metabolic characteristics, small colony variants often present a challenge to clinical laboratory microbiologists. The result is that these phenotypic variants are often misidentified leading to improper treatment or diagnosis. An alternative method to identify these small colony variants might be to look for molecular biomarkers that they have in common. Thus, molecular biomarkers for SCVs as we have observed here could be a valuable alternative tool for identifying *E. coli* SCVs. Additional biomarkers should be examined.

Figure 3.**Gel electrophoresis comparing expression of cyclopropane fatty acid synthase (*cfa*), *wcaC*, and *wcaK* in WT and IH3.**



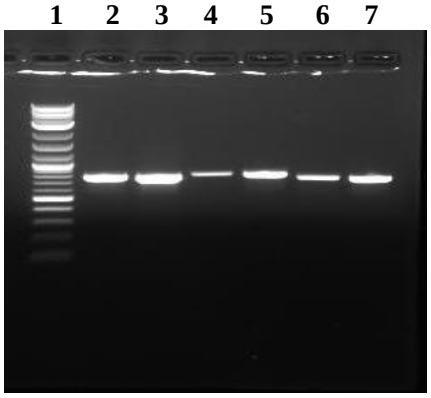
Lane 1 DNA Molecular markers (2- Log DNA ladder)
Lane 2 HfrH3000 *cfa*
Lane 3 IH3 *cfa*
Lane 4 HfrH3000 *wcaC*
Lane 5 IH3 *wcaC*
Lane 6 HfrH3000 *wcaK*
Lane 7 IH3 *wcaK*

Figure 4.Gel electrophoresis comparing expression of *gadA* and *fecR* gene in WT and IH3.



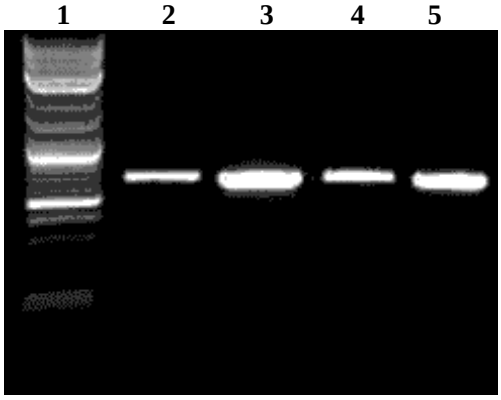
Lane 1 DNA Molecular markers (2- Log DNA ladder)
Lane 2 HfrH3000 *gadA*
Lane 3 IH3 *gadA*
Lane 4 HfrH3000 *fecR*
Lane 5 IH3 *fecR*

Figure 5.Gel electrophoresis comparing expression of cyclopropane fatty acid synthase (*cfa*), *wcaC*, and *wcaK* in BW7261WT and IH9.



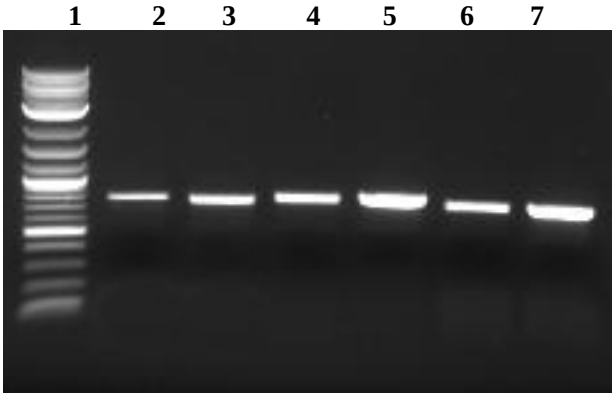
Lane 1 DNA Molecular markers (2- Log DNA ladder)
Lane 2 BW7261 *cfa*
Lane 3 IH9 *cfa*
Lane 4 BW7261 *wcaC*
Lane 5 IH9 *wcaC*
Lane 6 BW7261 *wcaK*
Lane 7 IH9 *wcaK*

Figure 6.Gel electrophoresis comparing expression of *gadA* and *fecR* gene in WT and IH9



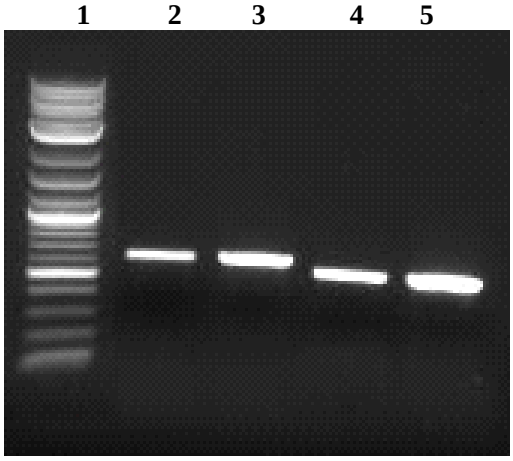
Lane 1 Mol Markers (2- Log DNA ladder)
Lane 2 BW7261 *gadA*
Lane 3 IH9 *gadA*
Lane 4 BW7261 *fecR*
Lane 5 IH9 *fecR*

Figure 7.Gel electrophoresis comparing expression of cyclopropane fatty acid synthase (*cfa*), *wcaC*, and *wcaK* in BW25113 and *lipA* mutant



Lane 1 DNA Molecular markers (2- Log DNA ladder)
Lane 2 BW25113 *cfa*
Lane 3 *lipA* mutant *cfa*
Lane 4 BW25113 *wcaC*
Lane 5 *lipA* mutant *wcaC*
Lane 6 BW25113 *wcaK*
Lane 7 *lipA* mutant *wcaK*

Figure 8.Gel electrophoresis comparing expression of *gadA* and *fecR* gene in WT and *lipA* mutant.



Lane 1 Mol Markers (2- Log DNA ladder)
Lane 2 BW25113 *gadA*
Lane 3 *lipA* mutant *gadA*
Lane 4 BW25113 *fecR*
Lane 5 *lipA* mutant *fecR*

3.3 Gene expression in SCVs is markedly different from wild type gene expression

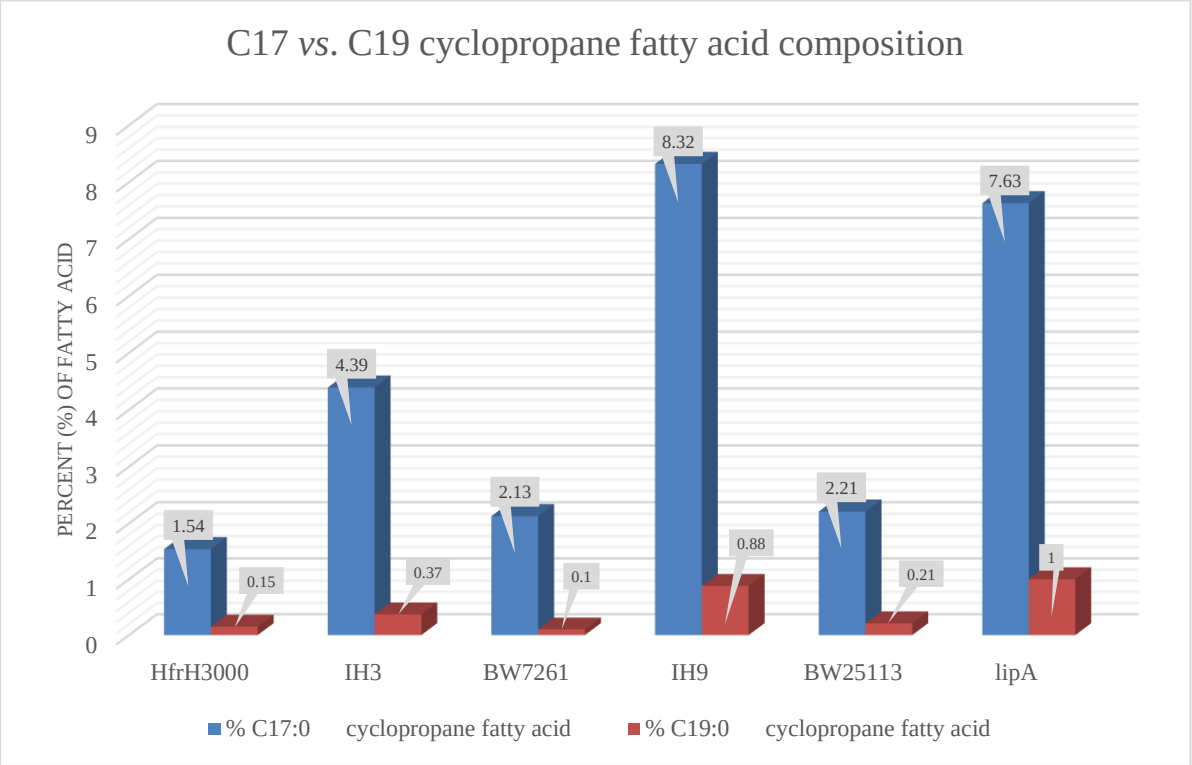
Upregulation of these genes is an indication of the propensity for biofilm formation, an increase of iron intake, an upregulation of acid stress response systems, and membrane remodeling as a result of cyclopropane fatty acid synthesis.

3.4 Whole cell fatty acid profile and cyclopropane fatty acids

Whole cell fatty acid profile and cyclopropane fatty acids. Since the *cfa* gene appears to be induced in the SCV strains compared to wild type, it was of interest to determine if we could detect higher amounts of cyclopropane fatty acids in the SCVs as well. In the process of forming cyclopropane fatty acids, the cis-monounsaturated fatty acids C16:1 (palmitoleic acid) and C18:1 (oleic acid) are converted to the cis-9,10-methylene hexadecanoic acid (17:0 cyclo) and cis-9,10-methylene octadecanoic acid (19:0 cyclo). This conversion occurs post-synthetically in situ in both the inner and outer membranes of the cell and requires the action of the cyclopropane fatty acid synthase, which adds a methyl group from S-adenosylmethionine to the cis-double bond of the unsaturated fatty acid (14, 15, 16).

The data in Figure 9 show the results of a fatty acid methyl ester (FAME) analysis of all three pairs of WT and SCV strains. In each case the C17:O cyclo species was 3-4 fold higher in the SCV, and the C19:O cyclo species was about 3 to 8-fold higher in the SCVs. It is apparent the C17:O cyclo species was the more abundant of the two, as has been reported in the literature (16). It is significant that the cyclopropane fatty acids in the SCVs were elevated in log phase cells, because this normally occurs only at the onset of stationary phase.

Fig 9. Fatty acid methyl ester (FAME) analysis of all strains.



FAME analysis performed on cells grown in LB medium to log phase with shaking. Experiments performed in duplicate with the percent of the total fatty acid species in each analysis shown in graph.

Small colony variants (SCVs) have been known for over a century (1), and have been observed with a variety of genera of bacteria (1, 3, 5). In the past 25 years, the bulk of research has been conducted on staphylococci, especially, pathogenic *S. aureus*. SCVs have three important phenotypes in common. They are slow growing, have a colony size about 1/10 of the parental cells, and are auxotrophs. A prevalent type of auxotrophism is for hemin and menadione. These mutations result in an inability to synthesize the heme prosthetic group of the cytochromes, and this leads to a defect in electron transport. This type of auxotrophic mutant has been described as an electron transport defective type (1). Further studies on *S. aureus* has shown that thymidine auxotrophy in *S. aureus* leads to a decline in Krebs cycle activity, which results in down regulation of the electron transport machinery (13). Strain JW0623 is an *E. coli* strain that is auxotrophic for lipoic acid due to a mutation in the lipoic acid synthase gene (*lipA*). Studies on the *lipA* mutant strain indicate that it is an *E. coli* SCV that resembles an electron defective SCV of *S. aureus* (5). An important property of these SCVs is that the strains can be phenotypically rescued by adding the missing metabolite. For example, with the *E. coli*, *lipA* mutant, addition of 5 µg/ml of lipoic acid reversed the slow growth and other properties of the strain such as acid resistance and hydrogen peroxide resistance.

In contrast to the *lipA* mutant, IH3 and IH9 have not yet been found to be auxotrophic. They were tested with hemin, menadione and thymidine, but this exposure did not enhance the growth rate of these two strains. Nevertheless, IH3 and IH9 present as slow-growing strains that form tiny colonies. They have an atypical morphology (smooth, convex colonies) like the *lipA* mutant. They are stable mutants like *lipA*, and do not revert back to the wild type form upon repeated subculture. As noted by Johns et al, (18) not all SCVs present the auxotrophisms common to Staphylococcus spp. One good example is the SCVs that result from *S. aureus* treatment with Triclosan™ (19). Some SCVs have been shown to have antibiotic resistance (1, 12, 14, 16). With respect to electron-transport deficient SCVs, a common resistance is to aminoglycosides, because of a decrease in membrane potential (20). The *lipA* strain could not be tested for aminoglycoside resistance as the strain was received with an aminoglycoside resistance marker. Neither IH3 nor IH9 were resistant to kanamycin. None of the strains were resistant to tetracycline, chloramphenicol or ampicillin.

The fact that all five genes we examined in the three *E. coli* SCVs, *gadA*, *wcaC*, *wcaK*, *cfa*, and *fecR* were upregulated is significant. Perhaps this quintet would be sufficient to diagnose the presence of an *E. coli* SCV. The *gadA* gene codes for glutamate decarboxylase, which is involved in *E. coli* acid resistance (17). These results also indicate that these genes are not induced because the mutants IH3 and IH9 were selected in an acid environment. The *lipA* mutant was not, and, in addition, the strains were cultured in normal, neutral LB medium for the RT-PCR analyses. The genes *wcaC*

and *wcaK* are important because they are involved in the synthesis of the exopolysaccharide colanic acid (18, 19). The biosynthesis of colanic acid depends on a 19 gene cluster (19-21). Colanic acid has been thought of as a loosely binding saccharide that coats the bacterial surface in biofilms (18). However, Meridith et al. (19) showed that it can form a tighter connection to lipopolysaccharide (LPS) under the appropriate environmental conditions, and this form is defined as M-antigen. Danese et al. (18) showed that disruption of colonic acid production did not seem to be critical for attaching to an abiotic surface, but was necessary for robust biofilm formation.

As colonic acid is polyanionic, it may serve as a buffer against protons and provide protection against acid stress (19, 22-24). The *cfa* gene encodes the cyclopropane fatty acid synthase, and cyclopropane fatty acids have also been implicated in protection against acid stress (25, 26). *fecR* is involved in the regulation of iron dicitrate transport into *E. coli* (27).

Why is it important to devise a molecular approach to the identification of small colony variants? From a clinical perspective, SCVs can cause chronic and recurrent infections, and they can be difficult to diagnose (1, 4, 12, 14, 28, 29). In two clinical cases, infections by *E. coli* small colony variants were difficult to diagnose using standard clinical methods. In one there was a recurrent urinary tract infection (12) and in the other a periprosthetic joint infection (4). Thus molecular biomarkers for SCVs as we have observed here could be a valuable alternative for identifying *E. coli* SCVs. Additional biomarkers should be examined.

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