Interrogation of chemical changes on, and through, the bacterial envelope of *Escherichia coli* FabF mutant using time-of-flight secondary ion mass spectrometry

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Time-of-flight secondary ion mass spectrometry (ToF-SIMS) using a primary ion beam of (CO$_2$)$_{6k}$ was used to analyse chemical changes in the bacterial envelope of a fabF knock-out *Escherichia coli* strain. fabF is the gene coding for FabF, the enzyme involved in the elongation of FA(16:1) to FA(18:1) and has been associated with plasmid transfer that can lead to acquired multiantibiotic resistance. Comparison of the membrane composition between fabF mutant *E. coli* and wild type *E. coli* during the logarithmic and stationary growth phases at two culture temperatures (37°C and 30°C) revealed substantial depletion of FA(18:1) in the fabF mutant during logarithmic growth that resulted in a correlated reduction in FA(cp19:0) during stationary phase. While no clear temperature dependence on the effect of the fabF mutation was found, a reduction in cyclopropanation was observed at lower culture temperature in the wild type strain. Additionally, depth profile analysis revealed a ‘thickening’ of the lipid A layer on the surface of the bacteria during stationary phase and also the appearance of cyclic enterobacterial common antigen (ECA$_{CYC}$) below the surface of the bacteria upon the shift from logarithmic to stationary growth phase.

**KEYWORDS**
bacteria, *E. coli*, FabF, lipids, mass spectrometry, ToF-SIMS

1 INTRODUCTION

Antibiotic resistance is an increasing threat towards modern medicine. The development and spread of antibiotic resistance is driven by factors such as inappropriate prescription and sales of antibiotics, use outside the healthcare sector and bacterial intrinsic factors. Conjugation, the process of direct cell-to-cell DNA transfer between bacteria, is a particularly hazardous route through which bacteria can obtain multiantibiotic resistance. The conjugation process in Gram-negative bacteria consists of several steps where first the donor cell, which contains the plasmid and a conjugative pilus, recognises the recipient cell and the donor-recipient pair is brought together by retraction of the pilus. A mating bridge is then formed between the two cells through which the plasmid is transferred. When the transfer and replication of the plasmid have finished, the mating pair dissociates. Through conjugation, genes coding for adaptive traits, such as antibiotic resistance, which could prove beneficial under selective pressures, are shared between cells. A treacherous trait of the plasmid is its ability to code for several genes, opening up for the possibility for the cells to acquire multiresistance in just one sharing event. Other benefits with sharing DNA are increased recombination, larger variation and faster trait fixation.

A recent study performed at the University of Gothenburg by Alam et al. identified several mutations in *Escherichia coli* which lead to impaired plasmid transfer capabilities. These are of interest...
because they suggest potential targets to inhibit conjugation.⁶ A time-of-flight secondary ion mass spectrometry (ToF-SIMS) study was later performed by Dimovska Nilsson et al. where several of the mutations that were expected to show effects in the bacterial envelope, by impairment of fatty acid synthesis or production of structurally important proteins and peptides, were selected to explore their defects and gain insight into how they affect conjugation.⁷ ToF-SIMS is a surface sensitive technique with a proven record for being sensitive to lipid detection and is therefore well suited to investigate biochemical changes in the bacterial envelope.⁸ ToF-SIMS also has a record of being used in bacterial analyses to classify bacteria responsible for urinary tract infection, classify Bacillus strains, and has been proven to be especially sensitive to secreted molecules with roles as antibiotics or in signalling.⁹–¹⁴ ToF-SIMS has also been used to image antibiotics at subcellular scale in single bacterial cells.¹⁵

The advent of gas cluster ion beams (GCIBs) has increased the sensitivity of ToF-SIMS to analyse larger mass molecules of up to several thousand kDa.¹⁶,¹⁷ while maintaining good surface sensitivity and depth resolution.¹⁸ The benefits of GCIBs have been demonstrated many times not only on tissue samples such as breast cancer tissue, mouse heart and skin cancer tissue but also on bacterial samples where lipid changes during the stringent response in E. coli with and without the global signalling molecule ppGpp were studied.¹⁹–²²

In the ToF-SIMS study by Dimovska Nilsson et al., the mutated E. coli strains were analysed using a 40 keV GCIB with clusters of 

\[(CO_2)_6k\]

both on the surface to investigate any lipid changes observed in the outer membrane of the cells and depth profiled to probe parts of the bacterial envelope.⁷ The depth profiles allowed detection of the higher mass species lipid A, the anchoring point of the larger molecule lipopolysaccharide (LPS), at \(m/z\) 1796, and cyclic enterobacterial common antigen (ECACYC), a molecule found in the periplasm, at \(m/z\) 2428. It was also found that the \(fabF\) knock out strain was depleted in the cyclopropanated fatty acid FA(cp19:0), a surprising find because FabF is expected to be involved in the elongation of FA(16:1) to FA(18:1), where only a small effect was found, not cyclopropanation. Further, this effect was observed in cells grown at 37°C although FabF is reported to be largely responsible for elongation of FA(16:1) at lower temperatures.

In this study, we further investigate the influence of FabF on membrane composition. Bacterial arrays of wild type (WT) and \(fabF\) E. coli strains were analysed using ToF-SIMS. Cells were cultured at two different temperatures, 37°C and 30°C and analysed at both logarithmic (log) and stationary growth phase to investigate the temperature dependence of the findings and see if the effects were found at both points of the growth curve.

## 2 | EXPERIMENTAL

### 2.1 | Culturing of E. coli strains

A strain of E. coli with a knock-out mutation in the \(fabF\) gene (\(\Delta fabF::kan/F\)), that will be referred to as \(fabF\) mutant, and a WT control strain with a neutral mutation, that will be referred to as WT (Table 1), were cultured aerobically in LB medium supplemented with kanamycin (50 \(\mu g/ml\)) and tetracycline (10 \(\mu g/ml\)) to select for the plasmid. The cells were cultured overnight in a rotary shaker at either 37°C or 30°C for the stationary phase sample. The overnight culture was diluted and grown in the same medium in a rotary shaker at 37°C or 30°C to log phase (OD₆₀₀ = 0.5) for the log phase samples. In total, eight samples were taken: the \(fabF\) mutant and WT bacteria grown at two temperatures and at two growth phases.

### 2.2 | Preparation of E. coli samples for ToF-SIMS analysis

Before ToF-SIMS analysis, the cells were washed using a washing procedure reported in previous work.⁷ Using this procedure, the cells in each bacterial solution were washed in 150 mM ammonium formate (AF) three times before they were resuspended in 150 mM AF and spotted onto a clean Si-wafer. Each bacterial solution was divided into two, a washing solution and an analysis solution. For each spot, a new pipette tip was used which was first washed three times in the washing solution and then two times in the analysis solution before cell suspension from the analysis solution was dropped onto the Si-wafer. Two technical replicates from each culture were deposited on the silicon wafer. Visual inspection of the droplets indicated that the sample drops were several bacteria thick with small variation between the different samples. The droplets were left to air dry and before inserting the samples into the ToF-SIMS instrument; they were placed in a desiccator for approximately 30 min.

### 2.3 | ToF-SIMS analysis

The ToF-SIMS analyses were performed using a J105-3D Chemical Imager (Ionoptika Ltd, UK). The J105 is a nonconventional ToF-SIMS instrument that has previously been described in detail.²³,²⁴ In short, the J105 uses a semicontinuous primary ion beam that allows for use of large clusters as primary ion projectile while also generating shorter acquisition time. The J105 used in this work is fitted with a 40 keV GCIB, and in this study, an ion projectile consisting of \((CO_2)_6k\) was

| TABLE 1 | Escherichia coli strains included in this work, their genotype and the label used in figures and text throughout this work |
| Label | Strain | Genotype | Reference |
|-------|--------|----------|-----------|
| WT | HA14 | BW25113 ΔargC::Kan⁶/F′Tet⁸ | Alalam, 2018, bioRxiv |
| fabF | HA42 | BW25113 ΔfabF::Kan⁶/F′Tet⁸ | Alalam, 2018, bioRxiv |
used. To select clusters of desired size, an electromagnetic Wien filter was used which selects clusters ± 20%. Using large gas clusters like (Ar)$_n$ or, as in this work, (CO$_2$)$_n$, generates less fragmentation resulting in higher signal from molecular ions and higher mass species compared with using more traditional ion beams such as C$_{60}$. The J105 utilises a buncher to bunch the secondary ions as they are injected into a reflectron ToF-analyser, thereby creating a time focus that makes this instrument less sensitive to topographical differences resulting in consistent mass resolution and improved mass accuracy. Surface and depth analyses of the cells were performed in both positive and negative ion mode; however, here the focus will be on the data collected in negative ion mode where the fatty acid signals associated with the action of FabF should be most clearly observed. The primary ion dose density (PIDD) was kept below the traditional static limit to $9.22 \times 10^{10}$ ions/cm$^2$ over an area of $32 \times 10$ mm$^2$. A depth analysis was performed of an area of $0.5 \times 0.5$ mm$^2$ with an accumulated PIDD of $9.22 \times 10^{12}$ ions/cm$^2$. While the sputter rate through these samples is not yet known this would be equivalent to approximately 45 nm of Irganox 1010, a common organic standard for organic SIMS depth profiling. Data were acquired over the $m/z$ 60–2500 mass range. A representative mass spectrum, taken from stationary phase WT E. coli at 37°C is shown in Figure S1.

3 | RESULTS AND DISCUSSION

fabF is the gene coding for the enzyme FabF or β-ketoacyl-ACP synthase II. This enzyme catalyses the elongation of palmitoleic acid, FA(16:1), into cis-vaccenic acid, FA(18:1), through the type II fatty acid synthetic pathway which is the principal route for bacterial membrane phospholipid acyl chain synthesis.  FabF is also known to be involved in temperature regulation by increasing the amount of FA(18:1) in the membrane as the temperature of the growth environment is decreased. To investigate the temperature dependence, the fabF mutant and WT were cultured at 37°C and 30°C, and the surface of the bacteria in the array sample was analysed with ToF-SIMS. The cultures were analysed at both log phase, where the cell population grows exponentially, and stationary phase, where growth has stopped due to lack of nutrients, to investigate if the results were dependent on the growth phase of the cell cultures. The results can

*FIGURE 1* Single ion images of fatty acids FA(16:1) at $m/z$ 253.2 (7 ppm), FA(cp17:0) at $m/z$ 267.2 (8 ppm), FA(18:1) at $m/z$ 281.3 (8 ppm) and FA(cp19:0) at $m/z$ 295.3 (11 ppm) in logarithmic (log) and stationary (stat) phase. Analysis area $32 \times 10$ mm$^2$ and a primary ion dose density (PIDD) of $9.22 \times 10^{10}$ ions/cm$^2$. Bar charts of FA(16:1), FA(18:1) and FA(cp19:0) providing an alternative illustration of the variation in signal intensity between the different cell cultures. Signal intensity normalised to number of selected pixels and number of droplets (i.e., 2)
also be used to examine the previous observation where the fabF mutant showed significant reduction in FA(cp19:0) signal when compared with WT E. coli in the stationary phase.7

Figure 1 shows single ion images of fatty acids FA(16:1) at m/z 253.2, FA(cp17:0) at m/z 267.2, FA(18:1) at m/z 281.3 and FA(cp19:0) at m/z 295.3 coupled with bar charts that provide an alternative means of assessing the signal differences between the different ions in each bacterial droplet. Some evidence of sample charging is present, particularly in the log phase droplets, but the overall trends in signal variation between samples are greater than the charging induced signal variation. So as not to bias the comparison, the bar charts shown alongside the images use averaged signal levels from 6, 15 × 15 pixels, squares (three per replicate droplet) selected avoiding the droplet edge and areas of especially bright or dark pixels (high/low signal) and show the average signal per pixel for the selected ions from each bacterial type. It can be seen how first, in the WT the levels of FA(18:1) and FA(16:1) increased slightly at the lower temperature in both log and stationary phase and second that the levels of FA(18:1) are drastically lower in the fabF mutant. We expected a more dramatic change in the ratio of FA(18:1)/FA(16:1) at lower temperature,27 but it appears that in our growth medium and strain background this does not occur; even lower temperatures should possibly be examined.

The lack of FA(18:1) signal from the fabF mutant is expected because FabF is involved in the production of FA(18:1). It should also be noted that a higher signal intensity of FA(16:1) and FA(18:1) was observed for cells grown in log phase compared with stationary phase. This is because these species are converted into the cyclopropanated fatty acids FA(cp17:0) and FA(cp19:0), respectively, as the cells enter stationary growth phase. In the previous work, the same reduction in signal from FA(18:1) was not observed in the fabF mutant. It was hypothesised that only a small reduction in the signal intensity in FA(18:1) was found in fabF mutant because the FabF is more active at lower temperatures than the 37°C in which the cells were cultured at for that work. This hypothesis is tested in this study by culturing the cells at both 37°C and 30°C. Another hypothesis proposed in that work was that FabB, another enzyme capable of producing FA(18:1) with lower efficiency, was able to compensate for the loss of FabF to some extent. However, FabB’s ability to partially compensate for the loss of FabF would not explain why a different result was found in this study as FabB would be able to do the same here. Instead, we hypothesise that a secondary mutation occurred in the strain that was used in the previous study, possibly in FabB, which made FabB more effective than normal.

In our previous work where only stationary phase bacteria were analysed, it was also found that while the amount of FA(18:1) was just moderately affected by the deletion of the FabF gene, it appeared that the larger effect was found in the cyclopropanated fatty acid FA(cp19:0) signal. A significant depletion of FA(cp19:0) in the fabF mutant was found also in this work. In Figure 1, it can be seen how the signal intensity of this fatty acid is very low for both WT and the fabF mutant in the cells analysed in log phase. We can also see how both WT and the fabF mutant show a higher signal intensity of FA(cp19:0) in the cells cultured at 37°C, although the difference between the temperatures is clearer for WT due to the low signal found in the fabF mutant. Cyclopropanation of unsaturated fatty acids mainly takes place as the culture enters stationary phase.32 Cyclopropanated fatty acids are produced by methylation, using S-adenosylmethionine, of cis-unsaturated fatty acids, that is, FA(16:1) or FA(18:1).33 This could explain why a large effect is seen not only on the direct product of FabF, that is, FA(18:1) but also the product of a reaction where FA(18:1) is reactant, that is, FA(cp19:0).

The data indicate that, at the two temperatures used in this study, FabF plays a major role in the generation of FA(18:1) and is not only

![FIGURE 2](image-url)
active at lower temperatures as expected based on the results from the previous study.\(^7\) Strikingly, the difference in FA(18:1) signal between the fabF mutant and WT E. coli is much higher in log phase compared with stationary phase. This can be explained by taking into account the conversion of the FA(18:1) to the cyclopropanated FA(cp19:0), that is abundant in the 37°C WT sample, as the cultures enter stationary phase that will deplete the FA(18:1). The FA(cp19:0) signal is much higher in the WT compared with the fabF mutant as the mutant entered stationary phase with low FA(18:1). A notable effect of temperature on the conversion of the FA(18:1) to FA(cp19:0) is seen. A similar observation is made for the conversion of FA(16:1) to FA(cp17:0); however, this is unaffected by the loss of FabF (Figures 1, S2 and S3).

In addition to the surface analysis of the E. coli membrane, depth profiles were performed on the two strains, WT and the fabF mutant, where the cells were cultured in either 37°C or 30°C and analysed at both log and stationary phase. The depth profiles of fatty acids FA(16:1), FA(cp17:0), FA(18:1) and FA(cp19:0) were plotted and show how all fatty acids show the same general trend where the signal intensity shows a signal maximum in the first layer analysed, and thereafter the signal decreases as the membrane is eroded by the ToF-SIMS analysis (Figures S2 and S3). Although the absolute value of the signal maxima varied, the same trend was seen regardless of growth temperature or phase in the growth curve.

In the depth profiles of the previous study, the higher mass pseudomolecular ion of lipid A was detected just below the surface of the E. coli cellular envelope and ECA\(_{\text{cyc}}\) was detected further down in what was hypothesised to be the periplasm.\(^7\) Lipid A is the anchoring point of the larger membrane molecule LPS, an endotoxin, which is the main constituent of the outer leaflet of the outer membrane in Gram-negative bacteria.\(^34,35\) ECA\(_{\text{cyc}}\) is a molecule present in the periplasm and although the function of ECA\(_{\text{cyc}}\) is largely unknown, it is thought to be involved in maintaining the outer membrane’s permeability barrier.\(^36\)

Figure 2 shows depth profiles of lipid A at m/z 1796.1 and a bar chart of ECA\(_{\text{cyc}}\) at m/z 2427.7 with data from both WT and the fabF mutant, cultured at 37°C or 30°C and analysed at either log or stationary phase. Depth profiles of lipid A were also generated for Figure S5, showing depth profiles with signal normalised to highest intensity and absolute signal, which illustrates the trends in the different cultures. The depth profiles of lipid A show how 1. lipid A in cells cultured at 30°C has a lower signal intensity at the very surface of the membrane compared with cells cultured at 37°C (Figure S5) and 2. in log phase cells, the signal from lipid A is highest at the very surface and then decreases as the membrane is eroded (Figure S5). This is more clearly depicted in Figure 2a where the averaged signal for the log and stationary phase depth profiles has been compared. The data behind Figure 2 can be found in Figure S4. Comparing the absolute numbers (Figure S5c,d), it turns out that for each cell culture, the signal intensity at the very surface is approximately the same when comparing log with stationary phase. However, in the cells analysed in stationary phase, the signal intensity increases with depth until approximately layer 15 (i.e., PIDD of 1.38 \(\times\) 10\(^{12}\) ions/cm\(^2\)), whereas in the cells analysed in log phase, the signal intensity steadily decreases with depth after the first analysed layer. This observation could be due to the extensive reorganisation of the membrane structure that takes place as cells enter stationary phase.\(^37\)

Depth profiles of ECA\(_{\text{cyc}}\) were generated showing how ECA\(_{\text{cyc}}\) appears to be absent in log phase (Figure S6). While the signal is increased with depth until a signal maximum is reached at approximately layer 50 (i.e., a PIDD of 4.61 \(\times\) 10\(^{12}\) ions/cm\(^2\)) (Figure S6) in stationary phase. The increase is lower for WT cultured at 37°C, however, this strain has a higher overall signal (Figure S6). No obvious signal maxima can be found in log phase (Figure S6a,c). This finding is more clearly illustrated in Figure 2b where the average signals for the log and stationary phase data are compared. The data behind Figure 2 can be found in Figure S4. These results indicate that ECA\(_{\text{cyc}}\) is not produced or transported to the periplasm in log phase but only in

\[\text{(A)}\quad \begin{align*}
\text{WT} & \\
\text{Log phase} & \quad \text{FA(16:1)} \xrightarrow{\text{FabF}} \text{FA(18:1)} \\
& \quad \begin{array}{c}
37\degree C \\
30\degree C
\end{array} \\
\text{Stat phase} & \quad \text{FA(cp17:0)} \xrightarrow{\text{FabF}} \text{FA(cp19:0)} \\
& \quad \begin{array}{c}
37\degree C \\
30\degree C
\end{array}
\end{align*}
\text{fabF} \\
\text{Log phase} & \quad \text{FA(16:1)} \xrightarrow{\text{FabF}} \text{FA(18:1)} \\
& \quad \begin{array}{c}
37\degree C \\
30\degree C
\end{array} \\
\text{Stat phase} & \quad \text{FA(cp17:0)}
\]

\[\text{(B)}\quad \begin{align*}
\text{Log phase} & \quad \text{Stat phase} \\
\text{Outer membrane} & \quad \text{Periplasm} \\
\text{ECA}_{\text{cyc}} & \quad \text{Lipid A} \quad \text{Phospholipid}
\end{align*}
\]

\[\text{FIGURE 3} \quad \text{(A)} \text{ Schematic of elongation and cyclopropanation seen in wild type (WT) and the fabF mutant in logarithmic (log) and stationary (stat) phase.} \quad \text{(B) Illustration of part of the bacterial envelope based on the findings of this work in log and stat phase.}\]
stationary phase. Previous results have shown that stationary phase cells have a less permeable membrane and this is regulated by the RpoS sigma factor.\textsuperscript{30} We hypothesise that the cyclic form of ECA is produced primarily in stationary phase and contributes to this strengthened impermeability. The RpoS dependence of this effect will be tested in future experiments.

A possible explanation for the temperature dependence of the cyclopropanation illustrated in Figure 1 is that the 30°C cultures grew slower compared with the ones cultured at 37°C and therefore reached stationary phase later. Although all the stationary phase cultures were expected to have reached stationary phase after overnight growth, it is possible that the growth temperature could have an effect on the time the cultures spent in stationary phase before they were analysed by ToF-SIMS, which could have an effect on the results. However, this is not supported by the depth profile analysis where the changes in the lipid A and ECA\textsubscript{CYC} signals (Figure 2) from log to stationary phase do not show a significant temperature dependence. There is the possibility that the lipid A and ECA\textsubscript{CYC} changes occur earlier in the onset of stationary phase compared with the cyclopropanation and this may be subject for further studies.

An illustrative summary of the results from the study is shown in Figure 3. Figure 3a shows that the WT \textit{E. coli} use FabF to elongate the FA(16:1) to FA(18:1), and this is independent of temperature in the range used in this study (37°C and 30°C). Upon reaching stationary phase, cyclopropanation of FA(16:1) and FA(18:1) occurs producing FA(cp17:0) and FA(cp19:0), respectively. A temperature effect was observed here where cyclopropanation was more extensive at 37°C compared with 30°C (solid versus dashed line). In comparison, the \textit{fabF} mutant shows severely reduced FA(18:1) generation during stationary phase and as such only FA(cp17:0) is produced upon the switch to stationary phase, but this again shows a mild temperature dependence. Figure 3b illustrates the switch in the density of lipid A in the membrane upon moving into stationary phase along with the appearance of ECA\textsubscript{CYC}. No temperature dependence on these changes was observed.

4 | CONCLUSIONS

ToF-SIMS has developed to become a powerful technique for analysis of biological samples such as the bacterial envelope of \textit{E. coli}. GCIBs, here coupled to a J105 instrument, have demonstrated once again that it is possible not only to detect high mass species and intact molecular ions on the very surface of a sample but also to depth profile them using GCIB ToF-SIMS.

A \textit{fabF} knock-out mutant strain was analysed and compared with a WT strain. The cultures were grown at 37°C and 30°C and analysed in log and stationary phase to investigate the temperature and growth phase dependence, respectively, on some of the FabF specific findings of our previous publication. In the previous work by our group, it was found how the amount of FA(18:1) was not significantly reduced in the \textit{fabF} mutant, even though it was expected to due to FabF’s involvement in the production of FA(18:1) through elongation of FA(16:1). Instead, the larger effect was found on the cyclopropanated fatty acid, FA(cp19:0).

In this work, an effect on the amount of fatty acids were found in both FA(18:1), contrary to what was found in the previous work, and in FA(cp19:0), in line with our previous findings.

In depth profiles of the \textit{fabF} mutant and WT, it was observed how lipid A, in log phase, showed a signal maximum at the first analysed layer compared with stationary phase where the signal maximum was found at approximately layer 15 (i.e., PIDD of $1.38 \times 10^{12}$ ions/cm$^2$). It was also found how ECA\textsubscript{CYC} was not present in log phase but appeared first in stationary phase. While this study showed clear trends that could be linked to biological processes, once improvements in the metrology concerning spot-to-spot signal variation and accurate measurement erosion rate are made, the approach may become applicable to detecting more subtle changes in bacterial membranes with improved relative quantitation.

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SUPPORTING INFORMATION
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