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Membrane staining and phospholipid tracking in *Pseudomonas aeruginosa* PAO1 using the phosphatidylcholine mimic propargyl-choline

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Keywords

Propargyl-choline, Cy3, Dye, Pseudomonas aeruginosa, membrane label, lipid tracking, phosphatidylcholine

Abstract

The use of membrane-specific dyes for *in vivo* fluorescent microscopy is commonplace. However, most of these reagents are non-specific and cannot track specific lipid species movement, instead often acting as non-covalent lipid associated probes or requiring uptake of whole lipids and acyl tails into the membrane. This issue has been solved in eukaryotic cell biology by use of click-chemistry liable phospholipid headgroup pulse-labels. Here we describe a method for *in vivo* phospholipid labelling by fluorescent imaging in *Pseudomonas aeruginosa* using a phosphatidylcholine (PC) mimic, “propargyl-choline” (PCho). This click-chemistry liable headgroup mimic is visible by microscopy and allows the covalent labelling of lipids. Fluorescence of the cell membranes, visible in heterogenous patches, is dependent on PCho

concentration and is localised in the membrane fraction of cells, demonstrating that it is suitable for membrane labelling and cell imaging.

Impact statement

Pseudomonas aeruginosa is an opportunistic pathogen. Pathogenicity and antibiotic resistance of the organism can be partly attributed to the presence of phosphatidylcholine lipids and more broadly the cell envelope. In 2019 more than 82,000 people died due to *P. aeruginosa* strains with resistance to one or more antibiotic treatments (1). In order to enable better study and understanding of *Pseudomonas sp.* lipids we describe an *in vivo* method to label *Pseudomonas aeruginosa* PC lipids and describe their subsequent visualisation by click-labelling and fluorescent microscopy. The phospholipid headgroup mimic propargyl-choline(PCho), substituting for a phosphatidyl choline headgroup(PC), has previously been used in mammals as a click-able microscopy label for use in membrane stability assays in engineered bacteria. Here we show its use in 'wild-type' bacterial cells, as a method to visualise the movements and localisation of membranes, similar to FM4-64 and applicable in situations where the tracking of a specifically labelled membrane lipid is useful. The ability to image a lipid mimic such as PCho in a model species such as *Pseudomonas aeruginosa* using PCho in bacteria could also, as in eukaryotes provide insight on lipid related organisations, growth and replication stages of bacteria in general not yet touched on.

Data summary

The microscope images, code and GIFs of lipid movement can all be found using an associated OSF data repository DOI: https://doi.org/10.17605/OSF.IO/ZGDFQ

SI Figure 1. Cell Viability and propargyl-choline concentration

SI Figure 2. GIF of lipid movement

SI Figure 3. GIF of lipid tracking

The authors confirm all supporting data, code and protocols have been provided within the article or through supplementary data files.

Introduction

The insertion and maintenance of lipids in the inner membrane and inner leaflet of the outer membrane of Gram-negative bacteria is not yet fully understood. Whilst labelling of cells with lipid probes has revealed lipid raft localisation, cardiolipin localisation (2,3,4), and changes in

phospholipid abundance over time, few methods can track covalently modified lipids movement. Fluorescence-labelling techniques for microscopy typically monitor lipid movement and localisation using probes able to detect specific lipid headgroups (3) or by labelling the lipid tail (5). The phosphatidylcholine (PC) mimic, propargyl-choline (PCho), has recently been used to label phospholipids in *E. coli* cells, which were modified to include phosphatidylcholine metabolism. PCho labelled cells were suggested to have PCho in the inner and outer membranes, however PC biosynthesis is not native *to E. coli* and the study did not focus on the labelling and imaging process (6). Here we chose to study the distribution of the phosphatidylcholine mimic in wildtype *Pseudomonas aeruginosa PAO1* due to this organism natively having phosphatidylcholine in the cell envelope.

Pseudomonas aeruginosa is an opportunistic pathogen, with pathogenicity as well as antibiotic resistance associated with its cell envelope structure (6). PC has been shown to be required for efficient infection, through use of PC as an inflammation facilitator and pre-cursor for lung damage (8). Similar pathogens also scavenge PC from the host (9) which may play a role in growth, and PC has been shown to be required for cytotoxin production in related *Pseudomonas sp* (10). Therefore labeling technologies that enable analysis of exchanges and dynamics of lipids, especially phosphatidylcholine, would be a valuable tool.

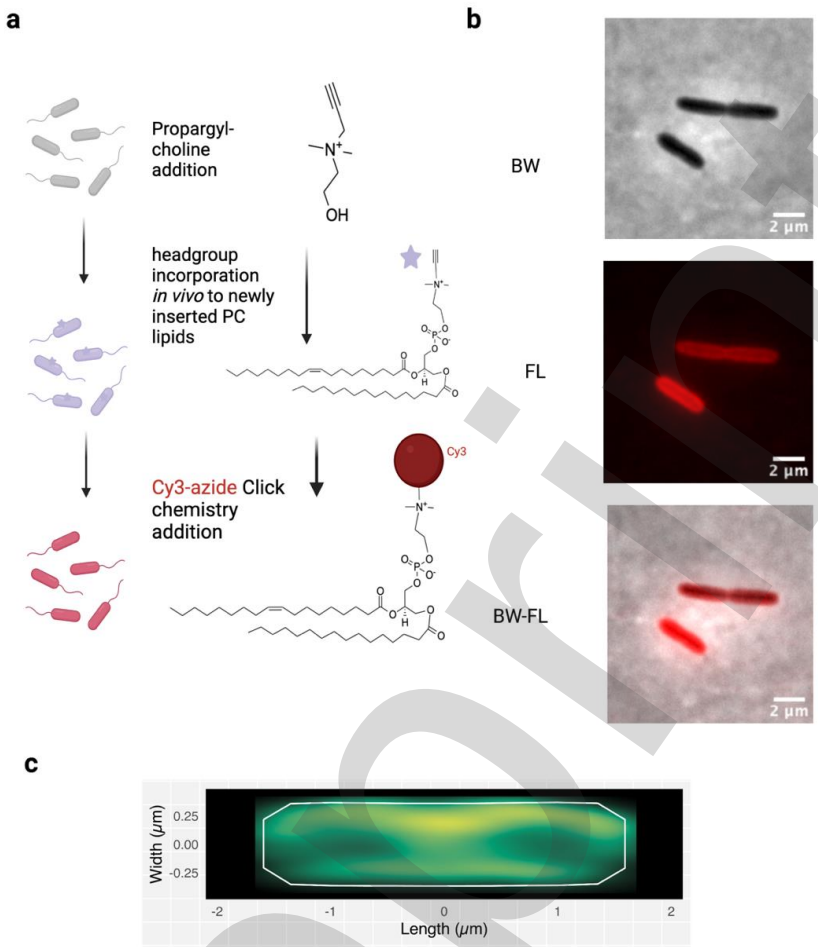
Mimics for phosphatidylcholine have been developed for mammalian studies. In the mammalian system, phosphatidylcholine metabolism can be directly supplemented with soluble choline mimics, which do not affect other aspects of core cell metabolism (11). PCho has yet to be used in wildtype Gram-negative bacteria for PC pulse-chase labelling. However, the capacity to label, image and track phospholipids in Gram-negative bacteria would enable this process of lipid insertion and dynamics to be studied simultaneously with peptidoglycan biogenesis through the simultaneous use of fluorescent *D*-amino acid mimics to address questions of how these processes are coordinated in the cell (12).

Therefore, in this study we establish the use of PCho (11) to determine localisation of the phospholipid phosphatidylcholine in *P. aeruginosa* after chemical crosslinking to an azide group. We found PCho to be of similar technical use to existing membrane labels such as FM464, however with potential for tracking this specific lipid species distribution and behaviour rather than displaying an affinity for detecting or binding to lipids in general (13). The use of PCho, a soluble headgroup rather than lipid tail addition also removes the need for membrane perturbation when using full phospholipid addition (14) or use of mutant cells with alternative enzyme pathways to wildtype (6).

Results

The phosphatidylcholine headgroup mimic propargyl-choline localises to *Pseudomonas* membranes

In order to determine the efficacy of PCho as a membrane localising PC mimic, the compound was incubated with *Pseudomonas aeruginosa* PAO1 cells during exponential growth for 5 minutes. This growth period equates to approximately 0.25 of the generation time in these growth conditions (15). Cells were then fixed and labelled with a fluorescent Cy3-azide, as established in mammalian cells (11). The cells were also labelled for teichoic acid as previously described (16) (Figure 1a). Cells incubated with PCho that underwent click-labelling demonstrated fluorescence at the membrane after click-labelling for 5 minutes. This indicates that the lipid head group mimics are localised to the membrane specifically (Figure 1b).



110

111 *Figure 1. Phosphatidylcholine Lipid insertion can be visualised by Propargyl- Choline*

112 **a** Propargyl-choline mimics phosphatidyl choline and can be labelled by click chemistry **b** Membrane localisation of
113 propargyl-choline-cy3 fluorescence. (100µM) **c** Average localisation of cell fluorescence maxima 1119 cells, 0.1 OD
114 exponential phase by BACTmap (17)

115

116 We then optimised PCho staining across a variety of concentrations (SI Fig1), with 100 µM being
117 the optimum concentration for membrane visualisation. However, averaging of fluorescence
118 distribution using BACTmap software (17) (Figure 1c) revealed no specific increase in
119 fluorescence at any region except for the membrane and membranous cell division site.
120 Titration of PCho revealed no fluorescence above background in the absence of PCho and
121 visible fluorescence in cells from 1 µm to 100 µM PCho (SI Figure 1b).

122 The optimum insertion time was 5 minutes at 100 µM PCho, which was sufficient to visualise
123 membranes after washing. However, lower concentrations were also sufficient for visualisation

(SI Figure 1b). In order to determine whether PCho, and its storage buffer DMSO had a detrimental effect on growth, we measured the growth cycles of cells with higher PCho concentrations (SI Figure 1b). Only concentrations above 150µm PCho, corresponding to 2.5% DMSO, affected growth rate. This effect was marginal and was insignificant during growth phase. This indicated that at the PCho concentrations used for microscopy (1 µm – 100 µM) the growth defect was insignificant.

We then confirmed incorporation of PCho into the membrane by thin layer chromatography (TLC) of the lipid fraction prepared from whole *Pseudomonas aeruginosa PAO1* cells. The fluorescent headgroup of propargyl-choline-cy3 was only incorporated in cells that had both been labelled with PCho and Cy3 after click-chemistry labelling. This indicates that PCho was incorporated into the lipid fraction of cells (Figure 2). Phosphomolybdic acid staining for lipid species revealed an additional spot by TLC for cells without Cy3 addition. This could potentially represent an unlabelled PCho phosphatidylcholine mimic (Black line).

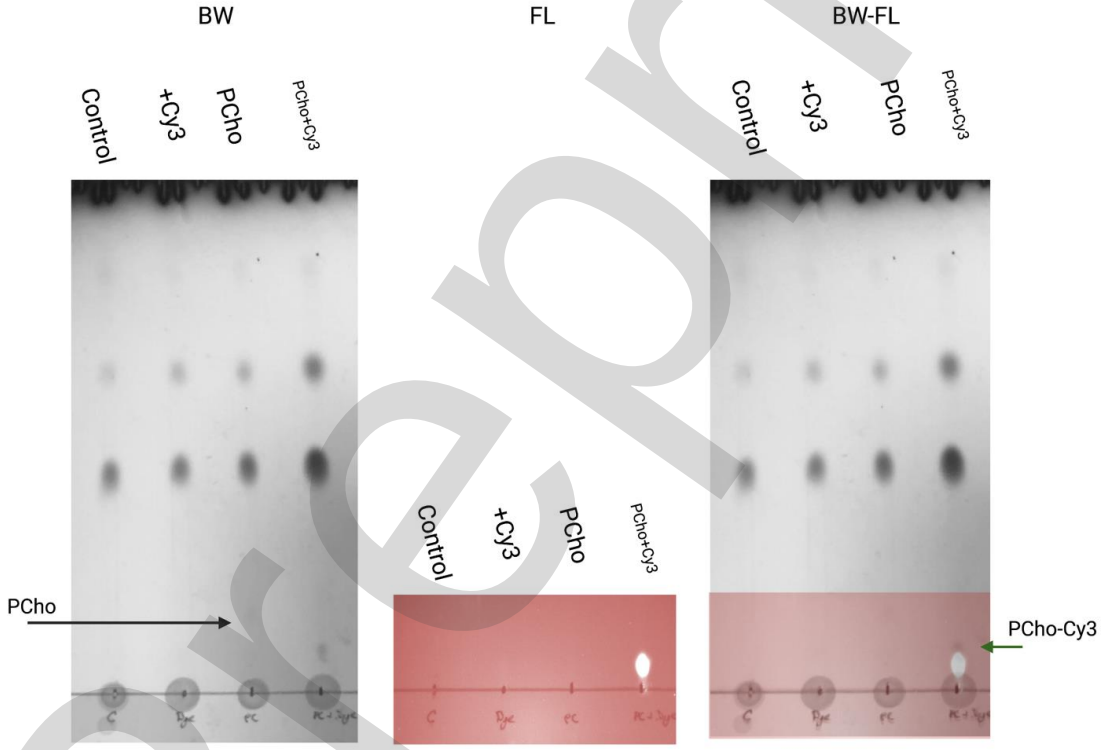


Figure 2 Insertion of propargyl-choline and Cy3 in *P. aeruginosa* membranes.

TLC plate of *Pseudomonas aeruginosa* PAO1 lipid fractions. All cells were subject to conditions used for click chemistry described in methods. Control - Wildtype *P. aeruginosa* lipid extract, +PCho 100 µM propargyl-choline addition, +Cy3 with Cy3 addition, PCho and Cy3, both propargyl-choline and Cy3 added. BW- Visible light photo of

stained TLC plate, FL- Cy3 fluorescence indicating Cy3 lipid attachment to lipid fractions Black arrow - propargyl-choline labelled lipid peak, Green arrow– Cy3-Propargyl-choline-lipid peak.

In order to investigate fluorescence distribution over larger cell stretches we labelled elongated cells. Cell elongation was achieved by exposure to the β-lactam antibiotic aztreonam, which is highly selective for inhibition of cell division associated PBP3 (FtsI) (18). We hypothesised that if PC is incorporated into the membrane at distinct sites then this could be visible in elongated cells. We found that labelled cells have regions of higher intensity fluorescence, which could indicate sites of increased PC insertion compared with no insertion over this same time period (Figure 3). Whilst we found differences in fluorescent labelling, with banded regions of higher PCho concentration along the non-dividing cells, this banding was not observable for untreated cells.

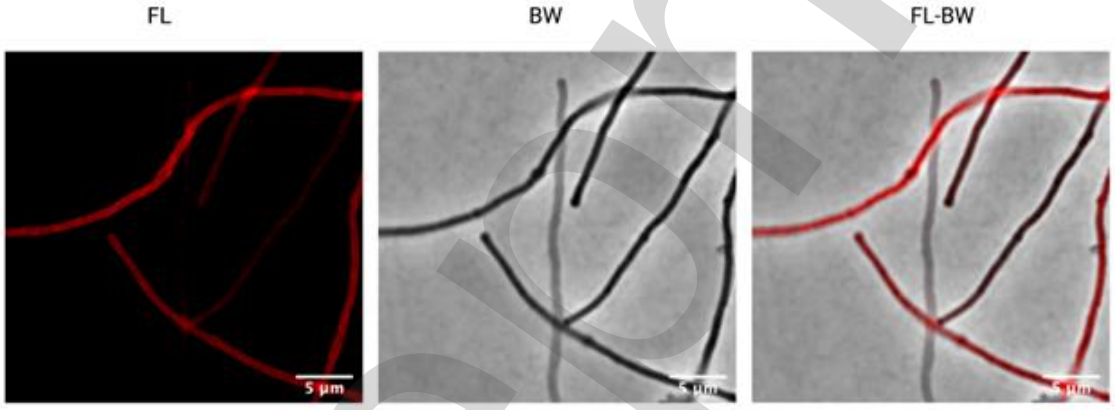


Figure 3. Aztreonam elongated cells show localised fluorescent incorporation

BW- Confocal, FL- fluorescent wavelength, BW-FL - merged Confocal Fluorescence images. All images were brightness and contrast adjusted manually for the highest-level detail observable in fluorescence channels. 5-minute incubation at 100 µM propargyl-choline

Propargyl-choline-Cy3 fluorescence has a similar localisation pattern to FM-464X

In order to evaluate Cy3-mediated fluorescence of labelled PCho as a general membrane stain, we compared it with a widely used membrane localisation method, the membrane dye FM-464X (19) (Figure 4). We found that the FM-464X membrane localisation was a visually clearer membrane label than PCho (Figure 4). However, fluorescence was heterogeneous among cells that were stained with either FM-464X or PCho. This suggests that differences in fluorescent intensity patterns between cells could be dependent on preparation/visual depth as indicated

by maps of fluorescent maxima, which have a peak at the cell centre. However, heterogeneity in PCho labelled cells also occurred at the cellular membrane level indicating a varying distribution of label incorporation.

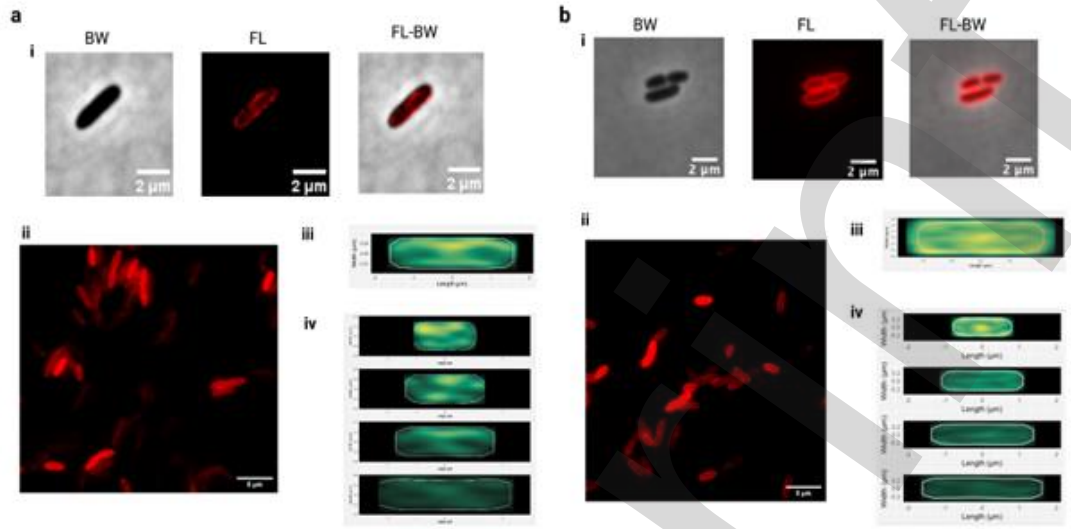


Figure 4. Membrane localisation and preference of Propargyl-choline labelled cells.

a Localisation of Cy3 fluorescence in propargyl-choline time-pulsed cells after 5 minutes incubation with 100µM propargyl-choline on individual cell i, groups of cells ii, and analysed as a population of 1119 cells across length classes iii/iv. **b** FM4-64X localisation on individual cells i, groups of cells ii and analysed as a population of 224 cells iii/iv.

The propargyl-choline click labelled lipids can track lipid movements.

We hypothesised that the PCho labelling methodology could be used to track lipid domains in live cells. The Cy3 click-labelled PCho photobleached over time, however the heterogenous patterns of fluorescence localisation changed over the course of miliseconds, independent of the bleaching effect. This suggested that the ~3% phospholipids that were potentially labelled could be changing location over the course of the imaging experiment. These groups of lipid particles could be tracked in ImageJ (20) using Trackmate (21), to give a population of potential lipid speeds over time. Lipid group track speeds revealed movement around a periplasmic track between 0.25-0.35 µm/s, which is similar to the speed of these lipids in mammalian cells (22) (Figure 5). Speed of group movement increased following exposure to 1 mM octanol, which is known to increase membrane fluidity (23) (Figure 5a). This indicates that tracking the lipid particle groups is indicative of lipid movement within the membrane. These results suggest that regions of increased fluorescence labelling could indicate lipid microdomains and lipid movements. Therefore this method could have potential uses in TIRF as a means of studying lipid movement phenomena.

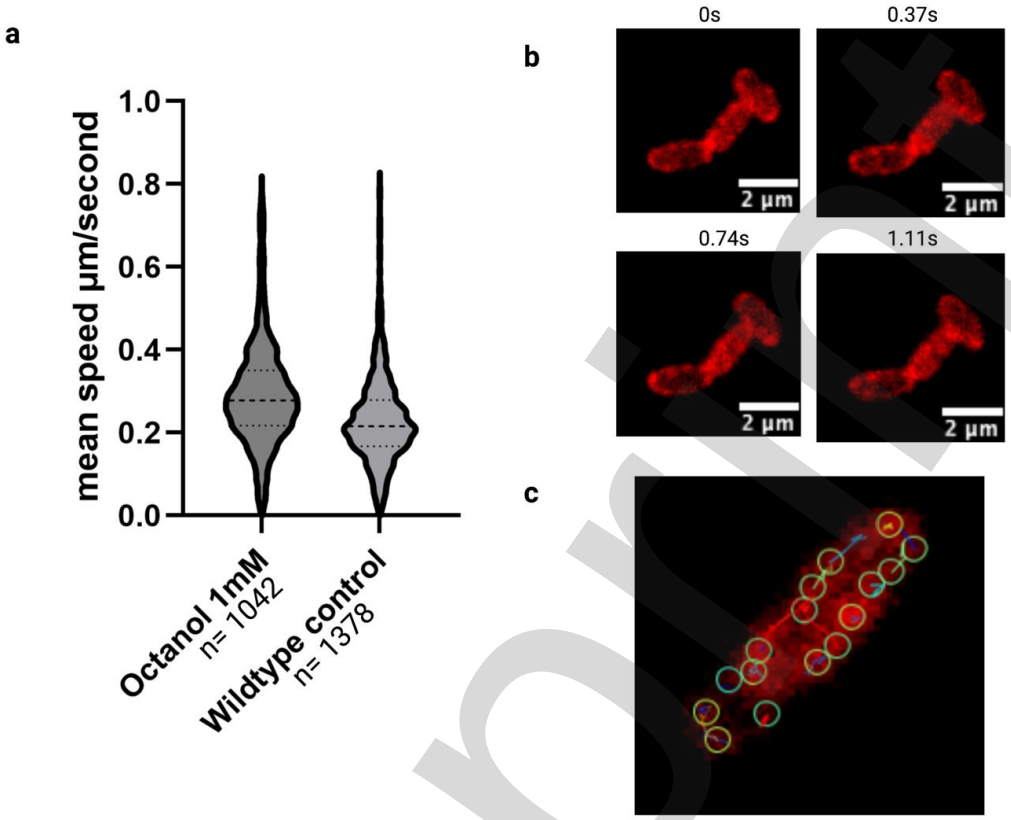


Figure 5. Use of Propargyl-choline as a potential lipid domain visualisation tool.

a Trackmate (21) mean track speed µm/s and fluorophore counts in a *Pseudomonas aeruginosa* PAO1 propargyl-choline and Cy3 labelled cells. T test difference in population, $p < 0.0001$ **b** 1.11s Timecourse of PCho-Cy3 fluorescence movements **c** attached GIF in supplementary data showing fluorescence change over time in periplasm. Red indicating fluorescence (SI Data https://drive.google.com/file/d/1t2L4Jn01psC8yq3H44j4XlmXO4NtEPLk/view?usp=drive_link), 100ms exposure Cy3 labelled PCho *Pseudomonas aeruginosa* cells **c** Trackmate fluorescence domains capture, and tracks shown

Conclusion

In this work, we have adapted an existing technique for phosphatidylcholine localisation in eukaryotic membranes for use in wild-type bacterial cells. Pulse-labelling *P. aeruginosa* cells with propargyl-choline (PCho) allowed for visualisation of new phosphatidylcholine mimic insertion in the cell membrane of living cells. (Figure 1). We confirmed insertion of the label

using extracted lipids from *P. aeruginosa* PAO1, which revealed a fluorescently labelled group visualised by TLC, only when both Cy3-azide and propargyl-choline were present during labelling.

This visualisation of a specific lipid, as opposed to other lipid labels which label lipids dependent on tail or have variant lipid affinities, allows for a specific and titratable bacterial membrane label. Analogous labels for use in cell envelope studies, such as peptidoglycan labelling with fluorescent d-amino acid mimics, have in the past been used in fluorescence microscopy allowing for significant advancements in the study of bacterial lifecycles (12). It should be noted that PCho incorporation could also be used for teichoic acid visualisation (16). Therefore, choice of a species without teichoic acids, such as *P. aeruginosa*, is necessary to allow for a direct lipid visualisation without potential crossover. Related species such as *Brucellus abortus* have been shown to use PC in their own membranes, after scavenging from the host eukaryotic cells, (9) therefore a marker for PC in bacteria would be useful for understanding these processes and the nuances of pathogenicity more clearly.

In this study, having tested this in *P. aeruginosa* PAO1*, we* propose this PCho incorporation and visualisation method as a phospholipid fluorescent labelling technique for compatible bacteria when other general labels are not suitable. We also propose the covalent label may have further use due to its titratable nature, in single molecule tracking techniques for study of lipid dynamics in bacteria.

Methods

Imaging of fluorescence in Pseudomonas aeruginosa

Strains were streaked from glycerol stocks onto LB Agar plates, and incubated at 37°C overnight. One PA colony was grown overnight at 37°C, at 180 rpm in 2 ml LB. The following morning a 1/10 dilution of the samples were then grown at 37°C, 180 rpm until the samples had all reached an OD_{600} of 0.3. 1% agarose in PBS was heated in a microwave until piping hot. Microscope slides were topped with a solution of 1% agarose in PBS which was flattened with a coverslip and left to cool. The coverslip was removed and 10 µl of sample was added to the slide, the coverslip was placed on top to spread the sample across the slide. Samples were then analysed using confocal microscopy specifically to identify the fluorescence, with corresponding filters dependent on expected fluorescence. Cy3-Propargyl-choline fluorescence was detected using a TXR filter set on a Leica DMi8 confocal microscope. The resulting images were taken in clusters of 15 across the sample at random to reduce bias, and allow for quantitive cell measurements.

Analysis of fluorescence localisation

Images were imported at LIFs or TIF libraries, which were then analysed by MicrobeJ software to determine determine cell contouring and maxima points. The points of increased fluorescence to background, were then tracked across the cell and mapped per individual point across thousands of cells, dependent on cell length. The points were then mapped for each

strain. Automatic, cellular counting and size determination by MicrobeJ (24) and BactMAP (17) allowed for quantitative analysis. Fluorescent points were tracked using custom tolerance and intensity filters maintained throughout study. Scripts and conditions for image analysis attached in the Supplementary Data file.

Propargyl-Choline click labelling

0.1 OD cells were incubated with alternative concentrations of propargyl-choline (dissolved in DMSO) (1µM to 2800µM) for 5 minutes. These cells were then concentrated by centrifugation for 10mins at 5000xg and (Figure 1/3/4) fixed by 4% paraformaldehyde PBS for 30 minutes, however this is not necessary for labelling (Figure 2/5). Cells were washed by centrifugation at 5000xg by pelleting, and resuspended in 100mM Tris-HCL pH 8.8, 1mM CuS04 50µl and ascorbic acid 0.1M . The cells were reacted with 100µM Cy3-azide, for a click chemistry reaction, then after 30mins at room temperature washed with TBS 1% Tween solution by centrifugation four times to remove the fluorescent azide remaining, before imaging.

Thin layer chromatography and lipid extraction

Lipid extraction used the Folch method (25) of lipid extraction, with 1:2:1 Chloroform:Methanol:Water at 55°C for 30mins, and vortexing, followed by extraction of the chloroform lipid phase after centrifugation. The thin layer chromatography was conducted on 60A Sepharose 254nm TLC plates, using 65:25:10 Chloroform, ethanol and acetic acid. The TLC plate fluorescence was recorded using Cy3 fluoremetric TXR filters on a 5x Zeiss Axio zoom microscope, and posed adjacently.

Tracking lipid foci

Trackmatev6.02 , implemented through a FIJI package was used to identify foci and track their movement over time (21). Cells observed in octanol 1mM were compared to H_20 and imaged at 100ms intervals using TXR filters. Trackmate parameters: Cell Threshold 60,000, foci diameter 0.1µm. linking distance 0.3µm, gap closing 0.3µm, Simple LAP tracking, LoG detector, subpixel localisation= True.

Author statements

Author contributions

CG performed click-labelling, culture, microscopy and analysis. CG and JB performed Thin Layer Chromatography of samples. The paper was written by CG, MB, JB and DR. The work was supervised by JB, MB, and DR.

Conflicts of interest

The authors declare no conflicts of interest

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