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Hung Ton-That *Editor*

The Bacterial Cell Wall

Methods and Protocols

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Preface

Unique to the bacterial kingdom, the peptidoglycan cell wall not only encases the cytoplasmic membrane and protects the cell from bursting inadvertently during growth and division under extremes of environments and conditions, but it is also responsible for, and maintains, the amazing variety of sizes and shapes (rod, coccoid, or spiral) that characterize the distinct classes of bacterial organisms. Not surprisingly, the cell wall has been the subject of an intriguing area of research in bacteria studied intensively for over eight decades. This began with a few model organisms such as *Staphylococcus aureus* and *Bacillus subtilis* for Gram-positive bacteria and *Escherichia coli* and *Salmonella typhimurium* for Gram-negative counterparts. The advent of genomics enabled the expansion of these studies to numerous bacterial species, most notably to many bacterial pathogens, addressing many fundamental questions involving the composition, biosynthesis, and regulation of bacterial peptidoglycan, and its role in pathogenesis and antibiotic resistance, by using a combination of genetic, molecular, biochemical, and cytological techniques. The purpose of this book is to provide current and future researchers with a compilation of many of the most important and useful procedures in a single resource.

In this book, we have curated a series of detailed protocols that employ advanced technologies as well as more conventional, yet quite effective and practical, methods to probe the cell wall, its many constituents, associated components, the processes, and the molecular machines involved in both Gram-positive and Gram-negative bacteria. In the first chapter, Hyland and colleagues describe a procedure involving bioorthogonal labeling of peptidoglycan in the Gram-negative anaerobe *Tannerella forsythia* by utilizing an azido-modified analog of N-acetylmuramic acid conjugated with a fluorescent dye via the powerful "click chemistry." Hu and Margolin demonstrate the utility of transmission electron microscopy coupled with tomography to visualize oligomeric structures of *E. coli* FtsA actin on membranes and their interaction with the bacterial FtsZ protofilaments. To et al. provide a simple method to visualize the cellular locations of a cell wall inhibitor protein in the relatively understudied Gram-negative anaerobe *Fusobacterium nucleatum*, while Kumar et al. employ computational and biophysical approaches to identify cell wall-associated modulators in *Salmonella enterica* serovar Typhi. The next two chapters detail experimental procedures of genetic manipulations in two emerging oral pathobionts, *Parvimonas micra* and *F. nucleatum*, aiming to facilitate future investigations of cell wall biogenesis. Zhang and Manley report their super-resolution method of single-molecule localization microscopy in conjunction with fluorescent D-amino acids to resolve bacterial peptidoglycan at the nanoscale level in both Gram-negative and Gram-positive bacteria. Focusing on the *S. aureus* lipoteichoic acid (LTA), Gründling and colleagues provide experimental procedures, employing western blotting and 2D nuclear magnetic resonance (NMR), to detect and characterize this accessory molecule. As a way to overcome the inherent problematic issues of plasmid-based expression, Bhat et al. detail a simple procedure of single-copy gene editing for a sortase-catalyzed pilus protein in *Actinomyces oris*. The next chapters from Cheung, Chang, Hall, and colleagues streamline their protocols to quantify the kinetics of sortase-catalyzed crosslinking reactions, to detect sortase by immunoelectron-microscopy, and to localize the remnant of a sortase-catalyzed substrate, respectively. Using X-ray crystallography, Tyagi et al. provide experimental procedures to determine structures of sortase-

catalyzed proteins. In the next two chapters, fluorescence microscopy is used to track cell wall-anchored proteins and to probe cell morphogenesis in *S. aureus*. In the penultimate chapter, Updegrove and colleagues cleverly repurpose the assembly process of spores in *B. subtilis* to generate a versatile platform for the display of ligands or antigens for the site-specific delivery of cargo or vaccines. Lastly, Xie et al. employ cryo-electron microscopy to determine a 3D structure of an enzyme involved in the synthesis of surface glycans in the tooth decay-causing Gram-positive bacterium *Streptococcus mutans*.

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Chapter 1

Bioorthogonal Labeling and Click-Chemistry-Based Visualization of the *Tannerella forsythia* Cell Wall

Stephen N. Hyland, Sreedevi Chinthamani, Sushanta Ratna, Kimberly A. Wodzanowski, Liam-Michael D. Sandles, Kiyonobu Honma, Catherine Leimkuhler-Grimes, and Ashu Sharma

Abstract

The objective of this chapter is to provide a detailed protocol for the peptidoglycan (cell wall) labeling of the periodontal pathogen *Tannerella forsythia* and the development of a laboratory-safe *Escherichia coli* strain utilizing the *N*-acetylmuramic acid recycling enzymes AmgK, *N*-acetylmuramate/*N*-acetylglucosamine kinase, and MurU, *N*-acetylmuramate alpha-1-phosphate uridylyltransferase, from *T. forsythia*. The procedure involves bioorthogonal labeling of bacterial cells with an azido-modified analog of the amino sugar, *N*-acetylmuramic acid, through "click chemistry" with a fluorescent dye. The protocol is suitable for the generation of fluorescently labeled peptidoglycan molecules for applications in the study of bacterial and peptidoglycan trafficking in the host cells and cell wall recycling in complex microbiomes.

Key words *Tannerella forsythia*, Peptidoglycan, Click chemistry, Cell wall recycling, Bioorthogonal, Microscopy, Mass spectrometry

1 Introduction

The human oral microbial community comprises over 700 bacterial species [1]. Colonization by *Tannerella forsythia* and other key species can cause microbial dysbiosis, leading to the development of periodontitis, an inflammatory disease that often leads to tooth loss and increases the risk for many systemic diseases [2, 3]. *T. forsythia* is auxotrophic for the peptidoglycan amino sugar *N*-acetylmuramic acid (NAM) due to the absence of the uridine diphosphate (UDP)-*N*-acetylglucosamine 1-carboxyvinyltransferase, MurA and the UDP-*N*-acetylenolpyruvoylglucosamine reductase, MurB enzymes in the bacterium required for the de novo synthesis of NAM. The bacterium relies on scavenging NAM and peptidoglycan (PG) fragments from the cohabitating bacteria for its survival in the oral cavity. The PG

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scavenging activity of the bacterium is expected to modulate host immunity by limiting the availability of muropeptides which serve as ligands for the intracellular nucleotide-binding oligomerization domain-containing protein 2 (NOD2) receptors in immune cells. In addition, PG scavenging might impact the growth of cohabiting species and promote dysbiosis.

T. forsythia utilizes a novel transporter system comprised of two proteins, MurT (transporter) and MurK (sugar kinase), for the uptake of exogenous NAM [4] and its subsequent recycling by a salvage pathway (bypassing de novo synthesis of NAM) into PG biosynthesis via the action of NAM/NAG anomeric kinase (AmgK) and NAM α-1 phosphate uridylyltransferase (MurU) [5]. *T. forsythia* is thus an excellent organism for the incorporation of NAM analogs via this salvage pathway into the PG biosynthetic pathway. A suite of NAM probes has been developed with handles for bioorthogonal chemistry enabling labeling with fluorescent dyes, biotin, and photoactivatable crosslinkers [6]. In particular, an azide functional group has been used for "click"-mediated installation of fluorophores directly on PG. Recently, we reported labeling of the PG in *T. forsythia* with the azide NAM (AzNAM) probe and copper (I)-catalyzed azide-alkyne click chemistry (CuAAC) and strain-promoted azide-alkyne cycloaddition (SPAAC) with an alkyne fluorophore [7]. Additionally, AmgK and MurU homologs identified in *T. forsythia* were genetically transformed into an *E. coli* strain to create a model system for PG labeling. Herein, we provide step-by-step procedures for performing PG remodeling and labeling with the CuAAC and SPAAC click chemistry protocols and verification of the incorporation of the probe by mass spectrometry. In addition, we describe an easily adaptable and less fastidious *E. coli* model strain with heterologous expression of *T. forsythia* AmgK and MurU enzymes for standardization of PG labeling strategies and efficient production of labeled PG fragments for downstream applications.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 MΩ-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at 4 °C (unless indicated otherwise). *T. forsythia* ATCC 43037 strain used in this protocol can be obtained from American Type Culture Collection (ATCC.org) or the author A. Sharma on request. Follow all waste disposal regulations diligently when disposing waste materials and perform bacterial manipulation under Biosafety Level-2 regulation.

2.1 T. forsythia *Culturing*

1. Hemin: Dissolve 50 mg of hemin into 1 mL of 1 N sodium hydroxide and then add 99 mL of water. Store at 4 °C.
2. Vitamin K_1: Dissolve 50 mg of vitamin K_1 into 10 mL of 95% ethanol. Store at 4 °C protected from light.
3. *N*-acetylmuramic acid (NAM): Dissolve 100 mg of NAM in 100 mL of water. Then filter solution through a sterile filter (0.22 μm) and store in small aliquots at −80 °C.
4. Modified Brain Heart Infusion (MoBHI) medium: In 470 mL water in a 2-L Erlenmeyer flask, add and dissolve 15 g of brain heart infusion broth, 0.5 g of yeast extract, 0.5 g of L-cysteine, 5 mL of hemin stock and 0.1 mL of vitamin K_1 stock. Adjust pH to 7.2 and autoclave at 121 °C for 15 min. Transfer the autoclaved flask to a 45 °C water bath and leave the flask in the water bath for 2–3 h to cool. Then, inside a biological hood sterilely add 30 mL of room temperature equilibrated horse blood.
5. *T. forsythia* culture medium (TF medium) and agar plates: In a 2-L Erlenmeyer flask, add and dissolve 15 g of brain heart infusion broth (along with 7.5 g of agar if preparing agar plates), 0.5 g of yeast extract, 0.5 g of L-cysteine, 5 mL of hemin stock and 0.1 mL of vitamin K_1 stock. Adjust pH to 7.2 and autoclave at 121 °C for 15 min. Transfer the autoclaved flask to a 45 °C water bath and leave the flask in the water bath for 2–3 h to cool. Then, inside a biological hood sterilely add 30 mL of room temperature equilibrated horse blood and 5 mL of NAM stock solution into the medium. Shake the contents gently and pour the agar medium into Petri dishes (appx. 30 mL in 10 cm plates) and after the agar hardens, turn the plates upside down and leave plates at room temperature to dry and next day wrap plates and store at 4 °C. Medium can be aliquoted into smaller fractions in appropriate sterile containers and stored at 4 °C. Plates or medium can be used within 1 month after storage.
6. Anaerobic chamber: Set at 37 °C, equilibrated with an atmosphere of 85% N_2, 10% H_2, 5% CO_2.
7. Spectrophotometer with 1 mL glass cuvettes.
8. Sterile inoculating loops.
9. 2 L Erlenmeyer Flasks.
10. pH meter.
11. 10 cm Petri dishes.
12. Sterile filters (0.22 μm).
13. Heating water bath.
14. Screw cap glass tubes.
15. Centrifuge.

2.2 Bioorthogonal Cell Labeling with AzNAM and Fluorescent Detection of T. forsythia Cell Wall

1. NAM, 0.1 M stock. Store at −20 °C in aliquots of 25–50 μL volumes until needed.
2. AzNAM, 0.1 M stock: Dissolve 5.0 mg of AzNAM (MW = 334.285 g/mol) in 150 μL of sterile filtered water. Store at −20 °C in aliquots of 25–50 μL volumes until needed. AzNAM probe material can be synthesized using published protocol [7] or purchased commercially from TOCRIS.
3. 4% paraformaldehyde in PBS.
4. $CuSO_4$, 50 mM stock: Dissolve 12.5 mg of Copper(II) sulfate pentahydrate in 1 mL water. Store at 4 °C.
5. BTTAA, 7 mM stock: Dissolve 3.0 mg of BTTAA in 1 mL water. Store at −20 °C.
6. Fresh (+)- Sodium L-ascorbate solution, 12 mg/mL: Dissolve 12 mg of NaASc powder in 1 mL water. Prepare fresh each experiment (*see* **Note 5**).
7. Cy5-Alkyne, 1 mg/mL (Click Chemistry Tools). Store at −20 °C.
8. Screw cap glass tubes.
9. 1.5 mL microcentrifuge tubes.
10. Plate shaker.

2.3 Confocal Imaging

1. Tweezers.
2. Clear nail polish.
3. Confocal Microscope (e.g., Zeiss Axio Imager or Zeiss LSM 800).
4. Poly-L-Lysine solution, 1 mg/mL stock: 1 mg poly-L-Lysine dissolved in 1 mL water to make stock and stored at 4 °C. 100 μL of stock solution is added to 900 μL of MQ water to make 0.1 mg/mL solution to add directly to the slide to coat.
5. Zeiss™ Cover Glasses, High Performance. Coverslips are coated with poly-L-lysine as described below.
6. Fisherbrand™ ColorFrost™ Plus Microscope Slides.
7. ProLong™ Glass antifade mountant (e.g., Invitrogen). Store at 4 °C.

2.4 Generation of E. coli Model Strain Heterologously Expressing T. forsythia AmgK and MurU Enzymes

1. *E. coli* DH5α Competent Cells.
2. pB-TfKU plasmid DNA [7].
3. 2xYT Media.
4. 10 cm Petri dishes.
5. Cell spreader.
6. Heating water bath.
7. 37 °C incubator.

8. Kanamycin, 50 mg/mL stock.
9. Lysogeny Broth (LB) medium.
10. LB agar plates.
11. Glycerol.

2.5 Bioorthogonal Cell Labeling with AzNAM and Fluorescent Detection of DH5α-Tf-KU Cell Wall

1. Reagents 1–6 from *Materials 2.2*.
2. Fosfomycin, 10 mg/mL stock. Store at −20 °C.
3. Isopropyl β-D-1-thiogalactopyranoside (IPTG), 0.1 M stock. Store at −20 °C.
4. Alexa Fluor™ 488 Alkyne (Alk-488), 1 mg/mL. Store at −20 °C.
5. DBCO-488, 1 mM stock. Store at −20 °C.
6. 1× phosphate buffered saline (PBS).
7. Glass culture tubes.
8. 5 mL serological pipettes.
9. Benchtop centrifuge.
10. 1.5 mL microcentrifuge tubes.
11. Plate shaker.
12. Kanamycin, 50 mg/mL stock.
13. Lysogeny Broth (LB) medium.
14. LB agar plates.
15. Spectrophotometer and cuvettes.

2.6 Mass Spectrometry Confirmation of Peptidoglycan Remodeling

1. NAM, 0.1 M stock.
2. AzNAM, 0.1 M stock.
3. 50 mL conical tubes.
4. Fosfomycin, 100 mg/mL stock. Store at −20 °C.
5. Isopropyl β-D-1-thiogalactopyranoside (IPTG), 1 M stock. Store at −20 °C.
6. Lysozyme from chicken egg, 50 mg/mL stock. Prepare fresh each addition.
7. Digestion buffer: Add together 250 μL of 1 M Tris pH 7.9, 25 μL of 5 M NaCl, 20 μL of 0.5 M EDTA, and 4.75 mL of DI water.
8. Amicon Ultra 3K Filter Devices.
9. Lyophilizer.
10. Acquity UPLC BEH C18 column 2.1 × 50 mm (Waters).
11. Dionex UHPLC coupled to a Q-Exactive Orbitrap.

12. Water, Optima™ LC/MS Grade.
13. Acetonitrile, Optima™ LC/MS Grade.

3 Methods

3.1 T. forsythia *Culturing*

1. Streak an agar plate with a glycerol stock of *T. forsythia* cells and incubate the plate at 37 °C in the anaerobic chamber. It may take 4–5 days for colonies to appear.
2. Using a sterile bacterial loop transfer a loopful of colonies from the plate into 10 mL of TF medium dispensed in a screw cap glass tube; Rub the loop on the walls of the tube to dislodge and suspend the colonies into the medium.
3. Incubate the tube at 37 °C in the anaerobic chamber.
4. When the culture reaches an absorbance at OD_{600nm} of 0.2 (it may take 3–4 days) (*see* **Notes 1–4**), inoculate 1 mL of the bacterial suspension into 10 mL of fresh TF medium and incubate for 5 days in the anaerobic chamber at 37 °C as above.
5. At the end of the incubation the bacterial suspension should reach at least an absorbance of 0.5.
6. Pellet bacterial suspension by centrifugation at 8000 × *g* for 5 min at 4 °C.
7. Wash the cell pellet three times with MoBHI medium by resuspending and pelleting cells by centrifugation at 8000 × *g* for 5 min at 4 °C each time.
8. Resuspend cells in MoBHI medium to an OD_{600nm} of 1.0.

3.2 Bioorthogonal Cell Labeling with AzNAM and Fluorescent Detection of T. forsythia *Cell Wall*

1. Inoculate 50 μL of cell suspension from the above into 10 mL of MoBHI medium containing 2 mM AzNAM and incubate for 4–5 days or until the OD_{600nm} reaches 0.6 in the anaerobic chamber at 37 °C as above (**Note 6**).
2. Remove 1 mL of cell suspension into a 1.5 mL microcentrifuge tube (**Note 7**).
3. Pellet cells by centrifugation at 10,000 × *g* for 2 min at room temperature. Remove supernatant and wash cells twice with 750 μL of phosphate buffered saline, pH 7.4 (PBS) by repeated suspension and centrifugation.
4. Fix cells in 200 μL of 4% paraformaldehyde in PBS for 20 min. Pellet cells as above and wash twice with 200 μL of PBS.
5. Resuspend cells in 190 μL of PBS and add 4 μL of $CuSO_4$ (50 mM stock), 4 μL of BTTAA (7 mM stock), 4 μL of freshly prepared (+)- sodium (L) ascorbate (12 mg/mL stock), and 0.5 μL of Alk-488 or Cy5-Alkyne (1 mg/mL stock). Pipette up and down gently to mix.

6. Incubate cells at room temperature on a shaker for 30 min in the dark.
7. Pellet cells by centrifugation and wash cells three times with 500 μL of PBS each time.
8. Resuspend cells in 200 μL of PBS and incubate cells at room temperature in the dark for 30 min.
9. Centrifuge cells at 10,000 × *g* for 2 min and wash the cells once with 200 μL of PBS for final wash.
10. Resuspend the cells in 100 μL of PBS to prepare for microscopy. (**Note 8**).

3.3 Confocal Imaging

1. Add 200 μL of 0.1 mg/mL poly-L-Lysine to the center of each cover glass and keep it at room temperature for at least 3 h.
2. Remove poly-L-Lysine and wash 3 times with 500 μL of DI water.
3. Let it air dry for 15 min.
4. Spread about 10–15 μL of the labeled cell suspension to the center of a poly-L-Lysine coated slide using a plastic pipette tip.
5. Incubate at room temperature for at least 15 min in the dark to allow cell adherence. Remove extra liquid from the coverslip.
6. Add 5 μL of ProLong™ antifade mountant to the glass slide and using tweezers place coverslip inversely onto the glass slide making sure not to trap any air bubbles.
7. Incubate slides overnight at room temperature in the dark.
8. The next day, seal edges of coverslip with clear nail polish.
9. Image slides with a confocal microscope using a 63× oil objective. Fluorophore is visualized using the green laser line for Alk-488 (CuAAC labeling) and DBCO488 (SPAAC labeling) and the red laser line for Cy5-Alkyne (CuAAC labeling).
10. Confocal images of *T. forsythia* labeled cells from a typical experiment are shown in Fig. 1.

3.4 Generation of E. coli Model Strain Heterologously Expressing T. forsythia AmgK and MurU Enzymes

1. Thaw a 50 μL aliquot of *E. coli* DH5α competent cells on ice for 10 min. DH5α cells can be obtained commercially.
2. Add 2 μL of pB-TfKU plasmid DNA (5 ng/mL) that expresses functional AmgK and MurU enzymes of *T. forsythia* to the cells [7].
3. Incubate the cells on ice for 30 min.
4. Heat-shock the cells for 2 min in a 42 °C water bath.
5. Immediately place on ice for 2 min.
6. Add 750 μL of 2xYT media to the cells.
7. Incubate the cells shaking at 200 rpm and 37 °C for at least 1 h.

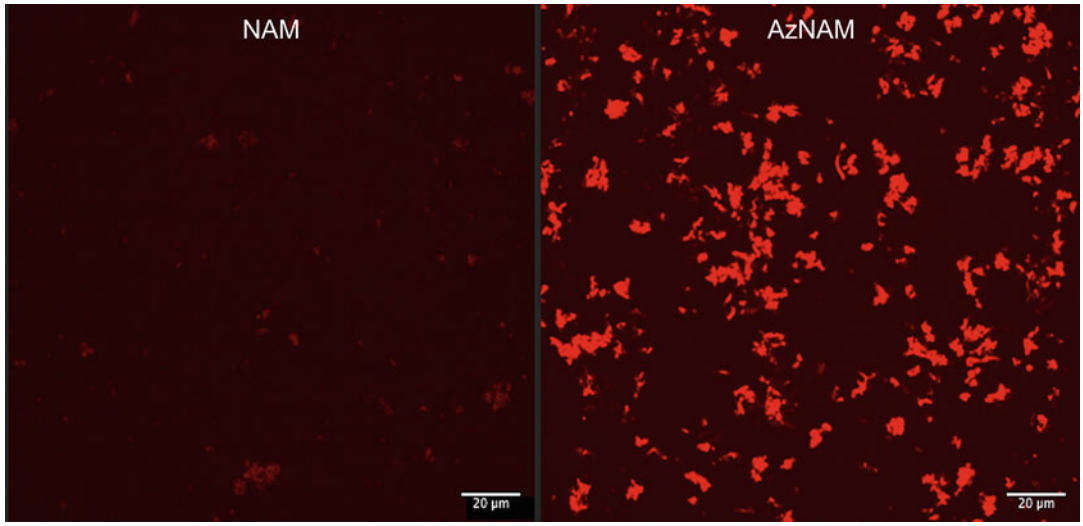

Fig. 1 Representative images of *T. forsythia* labeling. *T. forsythia* cells are fluorescently visualized following CuAAC click reaction with Cy5-Alkyne. NAM cells do not have the azide handle and therefore do not react with the fluorophores, leading to no fluorescence. Scale bars = 20 µm. (Reprinted with permission from Wodzanowski et al. [7]. Copyright 2023 American Chemical Society)

8. Spread 50 µL of cells over a kanamycin (50 µg/mL) LB agar plate using a glass spreader.
9. Incubate plate overnight at 37 °C.
10. Pick and grow 4–5 colonies from the plates and confirm the presence of plasmid by performing mini-plasmid isolation using a commercially available kit of choice (e.g., Qiagen). Positive transformants (DH5α-*Tf-KU)* should then be stored as 20% glycerol stocks at −80 °C.

3.5 Bioorthogonal Cell Labeling of DH5α-Tf-KU with AzNAM and Fluorescent Detection of Cell Wall

3.5.1 CuAAC Labeling

1. Streak a kanamycin (50 µg/mL) LB agar plate with *E. coli* DH5α-Tf-KU glycerol stock using a pipette tip. Incubate overnight at 37 °C.
2. Inoculate a single colony from the plate into 7 mL of LB medium containing kanamycin (50 µg/mL) to prepare an overnight culture of DH5α-Tf-KU cells.
3. The next day, inoculate 150–200 µL of overnight cultured DH5α-Tf-KU cells into 7 mL of fresh LB medium supplemented with kanamycin (50 µg/mL).
4. Shake the culture tube at 200 rpm at 37 °C for approximately 1 h or until the OD_{600nm} reaches around 0.600.
5. Using a 5 mL serological pipette, remove and place 1.2 mL of cultured cells into each 1.5 mL microcentrifuge tube labeled NAM and AzNAM.
6. Centrifuge tubes at 6010 × *g* for 5 min.

7. Remove supernatant and resuspend cells in 190 μL LB medium.
8. Add 12 μL of 0.1 M corresponding sugar stock (6 mM NAM or AzNAM final concentration), 4 μL of fosfomycin (10 mg/mL stock) and 2 μL of IPTG (0.1 M stock) to each of the cell samples.
9. Incubate the cells shaking at 37 °C for 1 h.
10. Centrifuge at >9000 × *g* for 2 min. All the remaining wash and centrifuge steps will be at the same rotation speed.
11. Remove the supernatant.
12. Resuspend the cells in 600 μL of PBS and centrifuge at >9000 × *g* for 2 min to wash the cells.
13. Repeat steps to wash the cells a second time.
14. Remove the supernatant and resuspend the pellet in 4% paraformaldehyde in PBS for 20 min to fix the cells.
15. Centrifuge at >9000 × *g* for 2 min.
16. Remove the supernatant.
17. Resuspend the cells in 200 μL of PBS and centrifuge at >9000 × *g* for 2 min to wash the cells.
18. Repeat steps to wash the cells a second time.
19. Remove the supernatant and resuspend in 190 μL of PBS to prepare for the CuAAC click reaction.
20. Sequentially add 4 μL of $CuSO_4$ solution (50 mM stock), 4 μL of BTTAA (7 mM stock), 4 μL of freshly prepared (+)- sodium (L) ascorbate (12 mg/mL stock), and 0.5 μL of Alk-488 (1 mg/mL stock).
21. Incubate cells at room temperature on an orbital shaker for 30 min covered with aluminum foil to protect from light.
22. Centrifuge at >9000 × *g* for 2 min.
23. Remove supernatant.
24. Resuspend in 600 μL of PBS and centrifuge at >9000 × *g* for 2 min.
25. Repeat steps twice, first washing with 600 μL and then with 200 μL of PBS.
26. Remove supernatant and then resuspend in 200 μL of PBS.
27. Let sit covered at RT for 30–60 min.
28. Centrifuge at >9000 × *g* for 2 min.
29. Remove supernatant.
30. Resuspend in 100 μL of PBS and spread 15 μL onto prepared coverslip for imaging.
31. Follow Subheading 3.3 for imaging. Confocal images of labeled cells from a typical experiment are shown in Fig. 2.

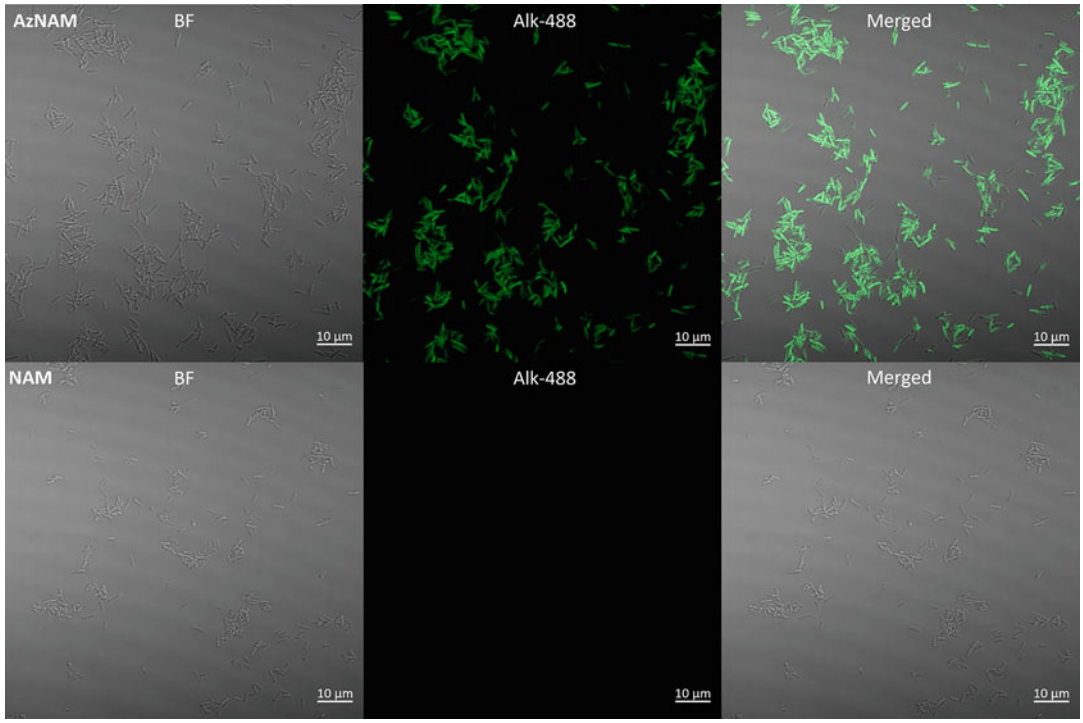

Fig. 2 Representative images of CuAAC labeling of DH5α-Tf-KU cell wall. Top row) AzNAM. Bottom row) NAM control. Confocal images were taken on a Zeiss LSM 800 microscope using Zen Blue. Alk-488 was visualized using the green laser line. BF = brightfield. Alk-488 = Alexa Fluor 488 Alkyne. Scale bars = 10 μm

3.5.2 SPAAC Labeling

1. Follow **steps 1–8** above in the CuAAC labeling protocol to remodel the cells with the NAM sugars.
2. Centrifuge at >9000 × *g* for 2 min.
3. Remove the supernatant.
4. Resuspend the cells in 600 μL of PBS and centrifuge at >9000 × *g* for 2 min to wash the cells.
5. Repeat steps to wash the cells a second time.
6. Resuspend cells in 194 μL of LB and then add 6 μL of 1 mM DBCO-488 (AZDye™ 488 DBCO; Click Chemistry Tools) to label the cells live with SPAAC.
7. Incubate cells at 37 °C covering with foil for 40 min while shaking.
8. Resuspend the cells in 200 μL of PBS and centrifuge at >9000 × *g* for 2 min to wash the cells.
9. Repeat steps to wash the cells a second time.
10. Remove the supernatant and resuspend the pellet in 4% paraformaldehyde in PBS for 20 min to fix the cells.
11. Centrifuge at >9000 × *g* for 2 min.

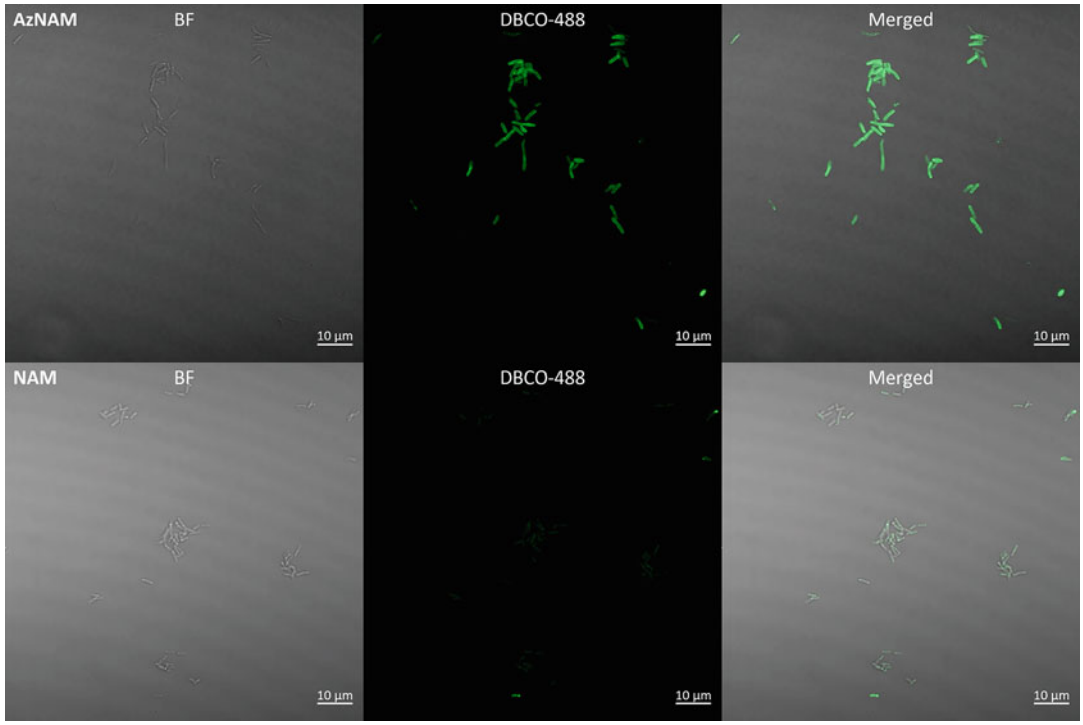

Fig. 3 Representative images of SPAAC labeling of DH5α-Tf-KU cell wall. Top row) AzNAM. Bottom row) NAM control. Confocal images were taken on a Zeiss LSM 800 microscope using Zen Blue. DBCO-488 was visualized using the green laser line. BF = brightfield. DBCO-488 = AZDye™ 488 DBCO. Scale bars = 10 µm

12. Remove supernatant and resuspend the cells in 600 µL of PBS and centrifuge at >9000 × *g* for 2 min.
13. Repeat steps twice, first washing with 600 µL and then with 200 µL of PBS.
14. Remove supernatant and then resuspend in 200 µL of PBS.
15. Let sit covered at RT for 30–60 min.
16. Centrifuge at >9000 × *g* for 2 min.
17. Remove the supernatant and resuspend in 100 µL PBS.
18. Spread 15 µL onto prepared coverslip for imaging.
19. Follow *Method 3.3* for imaging. Confocal images of labeled cells from a typical experiment are shown in Fig. 3.

3.6 Mass Spectrometry Confirmation of Peptidoglycan Remodeling

1. Streak a kanamycin (50 µg/mL) LB agar plate with *E. coli* DH5α-Tf-KU glycerol stock using a pipette tip. Incubate overnight at 37 °C.
2. The next day, inoculate 1 mL of overnight culture into 33.3 mL of fresh LB-kanamycin medium in a sterile 50 mL conical tube.

3. Remove 50 μL of freshly inoculated cells into a cuvette and determine OD_{600nm} using a UV-Vis spectrophotometer. Prepare blank using 50 μL of fresh LB. OD_{600nm} should be around 0.200.
4. Incubate culture at 37 °C for approximately 1 h or until the OD_{600nm} is about 0.600.
5. Centrifuge tubes at 3800 × *g* for 5 min.
6. Remove supernatant and resuspend pellets in 5 mL of LB medium.
7. Add 100 μL of 0.1 M corresponding sugar stocks, 20 μL of Fosfomycin (100 mg/mL stock), and 20 μL of 1 M IPTG to each of the cell samples.
8. Incubate the cells shaking at 37 °C incubator for 1 h.
9. Centrifuge cells at 3800 × *g* for 5 min.
10. Remove supernatant and resuspend pellets in 5 mL of PBS to wash.
11. Centrifuge at 3800 × *g* for 5 min. Repeat once.
12. Remove supernatant and resuspend pellet in 2.5 mL of digestion buffer (250 μL of 1 M Tris pH 7.9, 25 μL of 5 M NaCl, 20 μL of 0.5 M EDTA, and 4.75 mL of DI water).
13. Add 20 μL of freshly prepared lysozyme (50 mg/mL stock in DI water) to each sample.
14. Shake samples in 37 °C incubator for 3 days while adding 20 μL of freshly prepared lysozyme approximately every 12 h a total of 6 additions.
15. Centrifuge samples for 5 s, and add samples to Amicon Ultra 3K Filter Devices.
16. Spin sample to filter at 3000 × *g* for 30 min, and collect flowthrough.
17. Wash with 2 mL of water, and collect flowthrough.
18. Combine flowthroughs, freeze, and lyophilize overnight.
19. Dissolve lyophilized sample in a minimal amount of DI water (20 μL) and load onto an Acquity UPLC BEH C18 column 2.1 × 50 mm (Waters) using a Dionex UHPLC coupled to a Q-Exactive Orbitrap (Thermo Fisher Scientific). Elute with the following gradient:

    0.5 mL/min – linear gradient starting from 0% A to 50% B in 4 min.

    Eluent A – 0.1% formic acid in water.

    Eluent B – 0.1% formic acid in acetonitrile.

20. Measure the absorbance of the eluting peaks at 505 nm and further subject the peaks to high-resolution mass spectrometric analysis in positive ion mode; we utilize Q-Exactive Mass Spectrometer with Xcalibur Qual Browser. The lysozyme products are disaccharide fragments with various numbers of amino acids in the peptide chain off of the 3 position of NAM. Probe incorporation is confirmed by observing peaks that display the proper isotopic pattern with the observed mass within ±10 ppm of the expected mass. Representative analysis and confirmation is displayed in Figs. 4 and 5 for both NAM and AzNAM.

4 Notes

1. For preparing medium or agar plates, bring the temperature of the medium to 45 °C before adding blood to avoid lysis.
2. *T. forsythia* is a fastidious organism and a strict anaerobe. The quality of the horse serum and the correct composition of anaerobic gas mix is very important to obtain optimal growth. Prior to inoculation, medium should be well-equilibrated to the anaerobic environment.
3. Bacterial cells should be grown from cultures that were prepared from freshly streaked plates of lower-passaged frozen stocks.
4. To monitor the bacterial growth, take 1 mL of culture, pellet bacteria by centrifugation, wash cells once with water by suspending and pelleting cells by centrifugation. Finally, resuspend cells in 1 mL of water, transfer cell suspension into a glass cuvette and read absorbance at OD_{600nm}.
5. Previous experiments showed (+)-sodium L-ascorbate solutions not prepared fresh produced less effective click labeling.
6. The volume of culture medium for labeling can vary depending on the requirement.
7. Centrifuge rest of the labeled cells at 8000 × *g* for 5 min at 4 °C, wash cells twice with PBS. At this point cells can be used for downstream applications or frozen at −80 °C for purifying peptidoglycan and detection by fluorescent dyes and affinity probes.
8. If the pellet is small, cells can be resuspended in a smaller amount (25–60 μL). If the pellet is not visible, add 20 μL of PBS to the side of the tube away from the rotor axis where the pellet is expected to be.

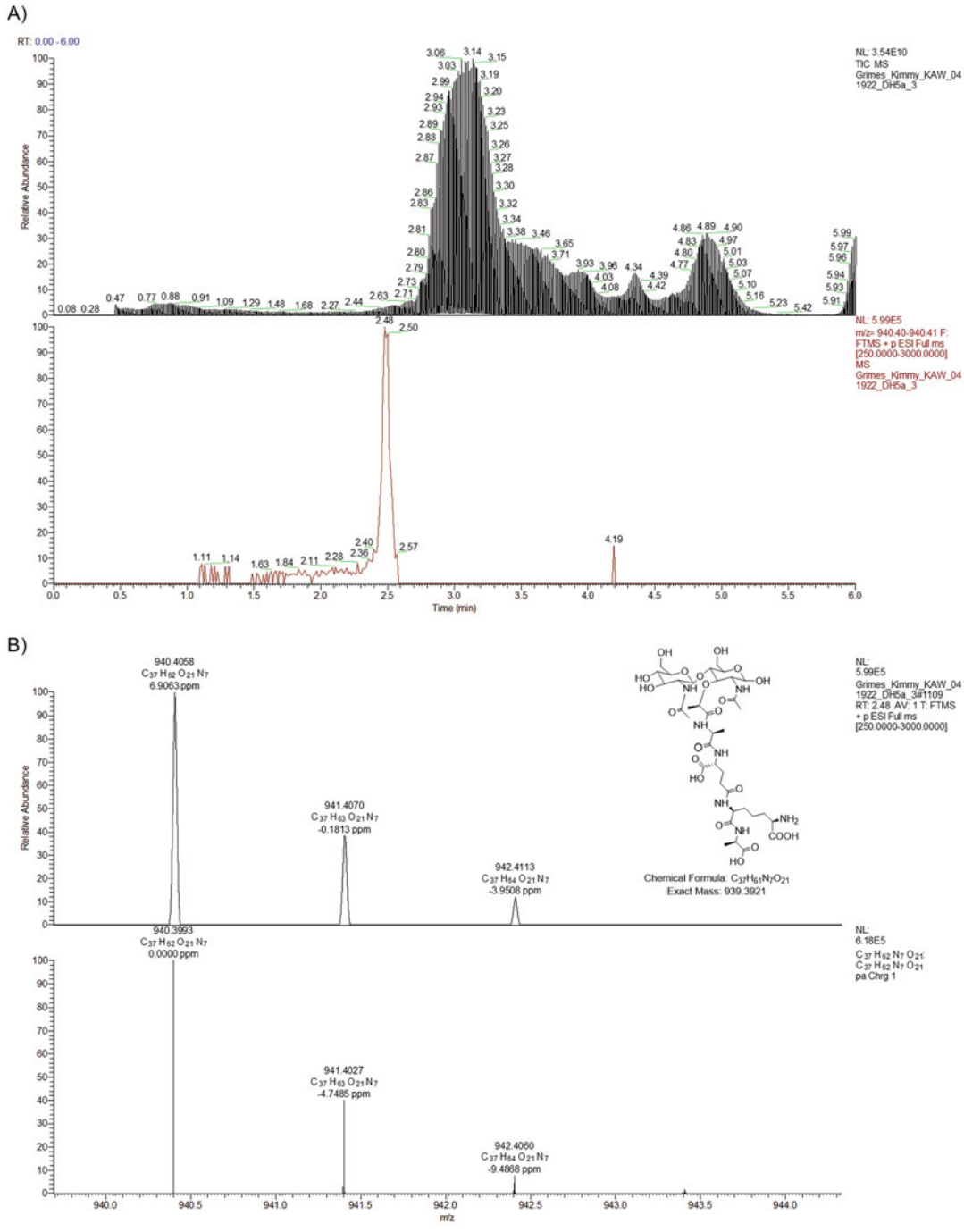

Fig. 4 Representative mass spectrometry results for NAM incorporation. (**a**) (Top row) Total ion chromatogram. (Bottom row) Mass range filter indicating relative abundance for corresponding NAM disaccharide mass. (**b**) (Top row) NAM sample isotopic peak distribution with corresponding structure of interest. (Bottom row) Simulated isotopic peak distribution using molecular formula of the structure of interest

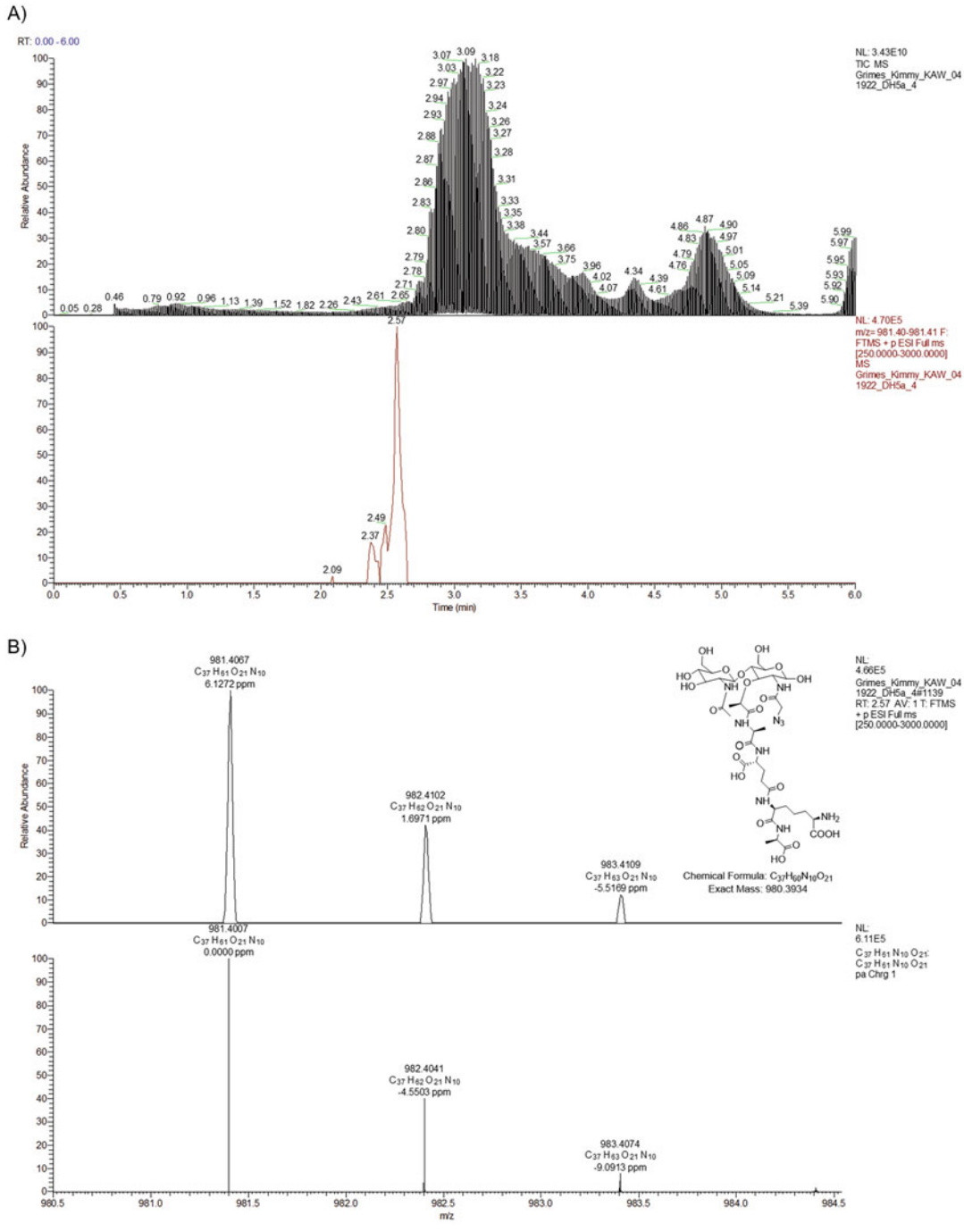

Fig. 5 Representative mass spectrometry results for AzNAM incorporation. (**a**) (Top row) Total ion chromatogram. (Bottom row) Mass range filter indicating relative abundance for corresponding AzNAM disaccharide mass. (**b**) (Top row) AzNAM sample isotopic peak distribution with corresponding structure of interest. (Bottom row) Simulated isotopic peak distribution using molecular formula of the structure of interest

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Chapter 2

Probing Membrane-Associated Cytoskeletal Oligomers of the Bacterial Divisome by Electron Microscopy and Tomography

Bo Hu and William Margolin

Abstract

The cell division machinery or "divisome" of many bacteria, including *Escherichia coli*, contains homologs of tubulin (FtsZ) and actin (FtsA) that interact with each other to promote the synthesis of septal peptidoglycan. FtsA oligomers have an essential role as a track for tethering dynamically treadmilling FtsZ protofilaments to the cytoplasmic membrane. Other bacterial cytoskeletal oligomers such as MreB also assemble on and move along the membrane. Structures of these oligomers on membranes in vitro may mimic their behavior in the cell. Here, we describe a protocol to visualize FtsA oligomeric structures on membranes and their interactions with FtsZ protofilaments using negative stain transmission electron microscopy along with tomography.

Key words FtsA, FtsZ, Cytoskeleton, Oligomers, Lipids, Monolayers, Electron microscopy, Tomography

1 Introduction

Transmission electron microscopy (TEM) has been crucial to visualize the polymerization behavior of cytoskeletal proteins in vitro, including bacterial FtsZ protein [1]. Like eukaryotic tubulin, FtsZ plays an important role in organizing key cellular processes, including cell division of bacteria [2]. The FtsZ family of proteins shares significant structural similarity with tubulins [3]. Despite this kinship, FtsZ does not form microtubules or even microtubule-like structures, but instead assembles into GTP-dependent single protofilaments visible by TEM both in vitro and in vivo [1]. These filaments congregate on the cell membrane in a tight band at the site of cytokinesis called the "Z ring" which is essential for cytokinesis to occur in most bacteria [2, 4].

Although FtsZ can polymerize, it is unable to bind to lipid membranes on its own. This interaction between FtsZ and

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membranes is crucial for assembly and function of the Z ring and requires FtsZ to bind to a membrane-associated protein [5]. In many bacteria including *Escherichia coli*, this partner protein is FtsA. Although FtsA shares some structural similarity with actin including the ability to form polymers [6], its association with the membrane, through a C-terminal amphipathic helix, results in a significant stimulation of oligomerization [7]. Lipid-bound FtsA structures include 20 nm-diameter "mini-rings" consisting of a curved single-stranded filament containing 12–13 FtsA monomers [8]. Various forms of FtsA form broken mini-rings (arcs) and straight double-stranded filaments [8, 9]. Addition of FtsZ to these various FtsA structures results in FtsZ filaments being tethered to the membrane via FtsA oligomers, mimicking the interactions in cells. Moreover, FtsZ protofilaments are kept apart by their association with FtsA mini-rings, but tend to bundle when the mini-rings switch to other oligomeric forms [8, 9].

Visualization of negatively stained purified FtsA and/or FtsZ on lipid monolayers or bilayers by TEM can directly measure these different oligomeric states in a biomimetic environment [7, 8, 10, 11]. Several other cytoskeletal polymers of bacteria such as MreB, which guides bacterial cell wall synthesis in rod-shaped cells [12] and SepF, which tethers the Z ring to the membrane in Gram-positive species [13], also associate with the cytoplasmic membrane.

As *E. coli* FtsA only forms detectable oligomers on lipids and is probably stabilized by its interaction with membranes, its purification is usually in the presence of membrane fragments [14]. The purifications of FtsZ and Histidine-tagged FtsA (His_6-FtsA) were described previously in detail [8, 15].

2 Materials

All buffers should be prepared with deionized MilliQ water. Store materials at room temperature unless indicated otherwise.

1. HPLC grade chloroform stock.
2. Sterile microcentrifuge tubes (1.5 mL).
3. Hamilton syringes, 10 μL and 100 μL capacities.
4. Amber borosilicate vials with PTFE lined lid.
5. Glass beakers.
6. Kimwipes.
7. Bath sonicator.
8. 100% ethanol.
9. Total *E. coli* lipids or polar lipids (Avanti Polar Lipids, Inc.), on ice, sealed with parafilm.

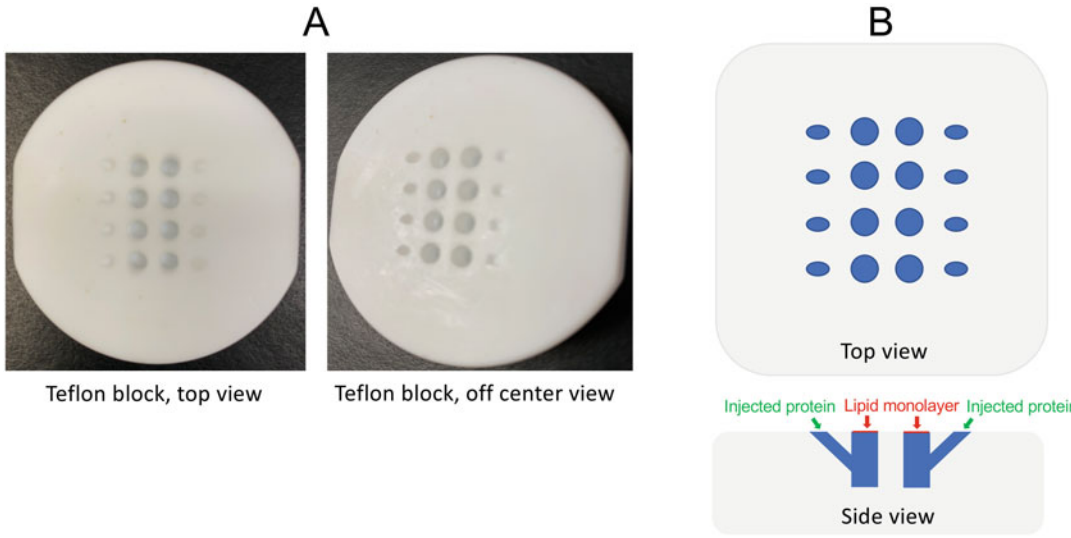

Fig. 1 The custom-fabricated Teflon block. (**a**) Photographs of top and off center of the actual block. (**b**) Top-view and side-view cartoons of the block and how it is used to inject protein samples underneath formed lipid monolayers

10. Teflon block (Fig. 1a, b), stored in 1:1 methanol:chloroform solution in a glass beaker in a fume hood.
11. Formvar/carbon-coated grids for electron microscopy, 200 mesh/nickel (Electron Microscopy Sciences), not glow discharged.
12. For optional Subheadings 3.6 and 3.7: Formvar/carbon-coated grids for electron microscopy, 200 mesh/nickel (Electron Microscopy Sciences), glow-discharged.
13. 10% SDS solution.
14. Plastic or glass petri plate with wet Kimwipe inside to maintain high humidity.
15. 0.1 M ATP stock, pH 7.5, store frozen.
16. 0.1 M GTP, pH 7.5, store frozen.
17. 1% uranyl acetate (in 1.5 microcentrifuge tubes in small aliquots). If crystals have formed, briefly spin down and use supernatant fraction for staining.
18. Pair of negative pressure tweezers (clamp by default, unclamp when squeezed).
19. Whatman #1 filter paper.
20. Storage buffer: 50 mM Tris pH 7.5, 250 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol.
21. Storage buffer without glycerol: 50 mM Tris pH 7.5, 250 mM KCl, 10 mM $MgCl_2$, 1 mM EDTA.

22. Affinity-purified His_6-FtsA [8] in Storage Buffer (thawed from −80 °C) + 0.05 mM ADP.
23. Purified FtsZ [8] in Storage Buffer (thawed from −80 °C) + 0.05 mM GDP.
24. Glow-discharged formvar carbon-coated nickel grids.
25. 300 kV-electron microscope (FEI Polara).

3 Methods

3.1 Preparation of Reusable Amber Borosilicate Glass Vials for Lipids

1. Soak glass vials and PTFE lined lids in 10% SDS overnight.
2. Clean them in a sonication bath.
3. Rinse thoroughly with deionized water.
4. Rinse 1× with 100% ethanol, let dry.
5. Rinse 1× with chloroform prior to use.

3.2 Lipid Preparation

1. Add ~5 mL of HPLC-grade chloroform to a small beaker.
2. Wash Hamilton syringes (10 μL and 100 μL volumes) by filling with chloroform in the small beaker and emptying into a larger beaker with a Kimwipe to absorb the chloroform (*see* **Note 1**).
3. Wash 3–5×, and dry any excess chloroform that remains on the tip with a Kimwipe.
4. Using the 100 μL syringe, add 249 μL of chloroform from a clean stock bottle (not the wash beaker) to the glass vial. There is no need to rewash this syringe, so it can be put away.
5. Using the 10 μL syringe, draw up ~3 μL of 25 mg/mL lipids (*see* **Note 2**), and depress plunger to the 2 μL line (drop will form at the tip but will not fall). Then touch this drop to the top edge of the chloroform in the vial without submerging the tip (*see* **Note 3**).
6. The remaining amount of lipids, plus any bubbles that are in the syringe, can be ejected into the beaker with a Kimwipe.
7. Mix the diluted lipids by flicking the tube or swirling the vial.
8. Rinse the 10 μL syringe 3× with the chloroform in the small beaker to remove any concentrated lipids from the tip.

3.3 Assembling Lipid Monolayers in Wells of a Teflon Block

1. Wash block with buffer needed to fill the wells. This will depend on the custom Teflon block (it is 80 μL for ours).
2. Suction buffer out with vacuum when done washing; use pipette to add/withdraw at injection port.
3. Add 90 μL of Storage Buffer lacking EDTA or glycerol to the top wells of the Teflon block.

4. Pump the 10 μL syringe up and down a few times in the diluted lipids, and draw up a higher volume than you need.
5. Dispense 2 μL at a time (the drop will stay on the tip of the syringe) and touch the tip to the drop of buffer.
6. Incubate in a humid chamber (petri plate, surround Teflon block with wet Kimwipes) for 1 h.
7. Place grids, shiny side down, on top of drops.

3.4 Adding Protein Samples to the Monolayers

1. Determine the volume of protein sample to be added (generally 5–10 μL), and remove that volume through the side injection well prior to adding the sample. This keeps the volumes in the wells consistent.
2. Add the protein sample to the side well (Fig. 1b), then slowly pipette up and down 10× using a volume of 5–10 μL (*see* **Note 4**). In the case of His_6-FtsA, 0.5 μM is a good final concentration.
3. Incubate 40 min, then add ATP (4 μM final concentration) and incubate 20 min.
4. If adding FtsZ to assemble on top of the lipid-bound FtsA, incubate 5 min then add GTP and incubate 20 min.

3.5 Negative Staining of FtsA on Lipid Monolayers with Uranyl Acetate

1. Spin aliquot of 1% uranyl acetate at top speed 1 min to pellet any crystals.
2. Draw up 5 μL in a pipette tip and push out to get a 5 μL hanging at the end of the tip.
3. Remove the grid with negative pressure tweezers (squeeze tweezers to open them, release to grip the grid with the tweezers) and use them to hold the grid sample (shiny) side up.
4. Quickly wick off any excess liquid with #1 filter paper (although it is OK if not all liquid is not off at this point and avoid blotting the grid excessively) and add the 5 μL drop of stain.
5. Wick off the first drop by touching a piece of filter paper to the side of the grid. Do not touch the sample area with the filter paper.
6. Leave on only for the amount of time it takes you to get the second 5 μL hanging drop of stain ready.
7. Add the second drop and incubate for 30 s.
8. Wick off the second drop. If there is any excess stain pooling under the tweezer arm, wick this away with the corner of the filter paper by touching it to the tweezer arm.

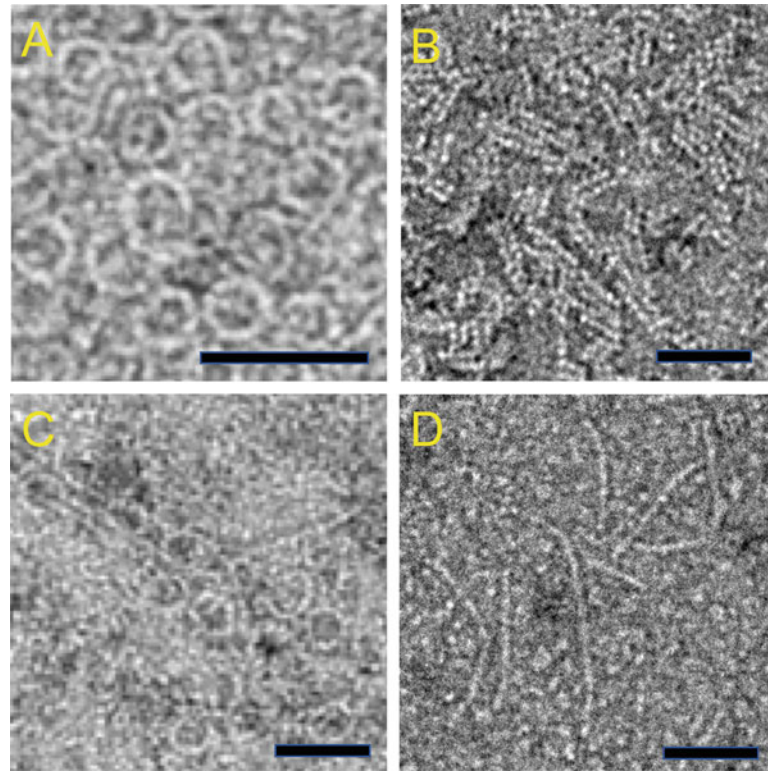

Fig. 2 Distinct oligomeric structures formed by FtsA and FtsZ on lipid monolayers by TEM. (**a**) Mini-rings formed by wild-type FtsA. (**b**) Double-stranded filaments formed by the FtsA variant $FtsA_{G50E}$. (**c**) FtsZ added after formation of mini-rings by wild-type FtsA. (**d**) GTP-induced FtsZ protofilaments in solution on glow-discharged grids. Scale bars, 50 nm

9. Allow the grid to dry (1–2 min) and place in a grid storage box (*see* **Note 5**). Label with date and any other relevant information that fits.
10. Image on a JEOL 1400 Transmission Electron Microscope coupled with a Gatan Orius CCD camera (*see* **Note 6**).
11. This method can distinguish different oligomeric structures of wild-type FtsA, which forms mini-rings (Fig. 2a), the $FtsA_{G50E}$ variant, which assembles into straight double-stranded filaments (Fig. 2b), or FtsZ added to lipid-bound wild-type FtsA (Fig. 2c).

3.6 Sampling Soluble Protein Within the Drop Not Associated with Monolayer (Optional)

1. After removing the grid with the monolayer, pipette up and down to mix the drop and transfer 60–70 μL to a microcentrifuge tube.
2. Incubate on ice until monolayer grids are finished staining.

3. Add 10 μL of these samples to glow discharged grids and incubate for 1 min.
4. Follow the staining procedure above.

3.7 Visualizing FtsZ Assembly in Solution Without Lipid Monolayers or FtsA

1. Incubate FtsZ (15 μM) with 1 mM GTP + 10 mM $CaCl_2$ in FtsZ Storage Buffer at 25 °C for 10 min.
2. Drop 10 μL of the sample on a glow-discharged formvar carbon-coated nickel grid and incubate for 1 min.
3. Wick away excess sample with filter paper.
4. Wash the grid with a 5 μL drop of 1% uranyl acetate, wick off.
5. Add another 5 μL drop of 1% uranyl acetate and stain for 30 s.
6. Wick stain away with filter paper, and allow the grids to dry.
7. Image as described above (Fig. 2d).

3.8 Tomographic Data Collection from Negatively Stained TEM Images

1. Image negatively stained samples on grids with a 300 kV-electron microscope equipped with a field emission gun and a direct detection device (Gatan K2 Summit).
2. Collect a single-axis tilt series at ~6 μm defocus with cumulative doses of ~200 $e^-/Å^2$ at pixel size of 4.5 Å, using the SerialEM tomography package [16].
3. For each dataset, collect 41 image stacks in a range from −60° to +60°, in increments of 3°. Each stack contains ~10 images.
4. Align each stack using Motioncorr [17] and assemble into drift-corrected stacks using TOMOAUTO [18, 19].
5. Align and reconstruct drift-corrected stacks by using marker-free alignment [20].
6. In total, 23 tomograms (3600 × 3600 × 120 pixels) were generated [8] (Fig. 3) and used for further processing.

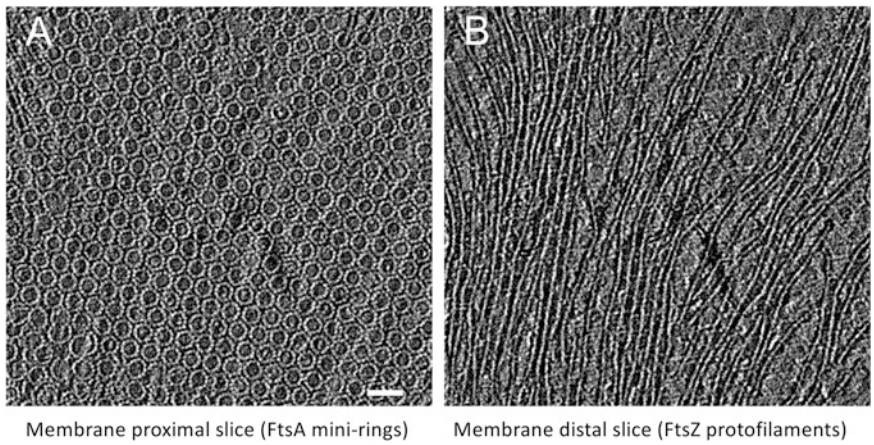

Fig. 3 Representative tomographic slices of a field of FtsA mini-rings (**a**) and FtsZ protofilaments on top of those mini-rings (**b**). The slice in (**a**) is closer to the lipid surface compared to (**b**). Scale bar, 40 nm

3.9 Sub-tomogram Analysis

1. Manually select ~200 particles (128 × 128 × 64 pixels) from 1 tomogram to generate an initial model by subtomogram averaging.
2. In the Krupka et al. study [8], the initial model was used to identify particles from 23 tomograms by template matching using PyTom [21]. In total, 18,400 particles were identified and extracted for subtomogram analysis using tomographic package I3 [22].
3. Define class averages after multiple cycles of alignment and classification (in the case of Krupka et al., two distinct class averages emerged).
4. Use IMOD [18] to take snapshots of 2D slices from 3D tomograms or 3D-averaged structures.
5. Segment and surface-render 3D-averaged structures using UCSF Chimera [23].

4 Notes

1. The plunger in a Hamilton syringe is fragile and needs to be perfectly straight – care should be taken not to bend it.
2. To remove lipids from stock on ice: unwrap the parafilm, open and remove what you need, then reseal quickly to minimize chloroform evaporation of the stock. Ideally, a small amount of nitrogen gas would be added prior to reclosing the vial to prevent oxidation, but *E. coli* lipids are robust, so it is OK to bypass this.
3. Do not submerge syringe tip into chloroform to avoid excess carryover. The amount needs to be *just* enough for the monolayer.
4. If adding smaller volumes (<5 μL) of, e.g., nucleotide to side wells of block, pipette up and down 20× to mix.
5. To deposit grid in storage box, 2 tweezers are often necessary. The first pair of tweezers hold the grid, while the second pair knocks the grid into the box.
6. Ideally, imaging should be done within 1 week of preparation.

Acknowledgements

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Chapter 3

Visualization of a Cell Wall Hydrolase Inhibitor in *Fusobacterium nucleatum* by Immunofluorescence Microscopy

Kevin To, Timmie Britton, and Hung Ton-That

Abstract

Innately present in tears, saliva and mucosal secretions, lysozyme provides a critical defensive strategy to the host by cleaving the β-1,4-glycosidic bonds between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues of peptidoglycan of invading bacteria, leading to bacterial lysis. To counter this class of cell wall hydrolase enzymes, bacteria produce several lysozyme inhibitors, a representative of which, MliC, was identified in *Escherichia coli*, *Pseudomonas aeruginosa*, and various bacterial species. The Gram-negative oral anaerobe *Fusobacterium nucleatum* encodes an uncharacterized lipoprotein homologous to MliC, whose localization is unknown. Here, we provide an experimental procedure to localize this MliC-like lipoprotein by employing immunofluorescence microscopy. In principle, this protocol can be used for any bacterial system to monitor protein localization.

Key words Immunofluorescence, Cell wall hydrolase, Membrane permeabilization, *Fusobacterium nucleatum*, Gram-negative bacteria, Lysozyme inhibitor

1 Introduction

Immunofluorescence microscopy (IFM) is a standard technique widely used to detect the presence, abundance, localization, and organization of biomolecules with high specificity in microbiological samples. For example, IFM was used to localize members of the RNA degradation & processing complex to a specialized cytoplasmic compartment [1] and cell division proteins FtsZ, FtsA, and ZipA to a cytoplasmic cell division ring in *Escherichia coli* [2]. This technique relies on the specificity between fluorescently conjugated antibodies and their cognate antigen. Briefly, bacterial cells are immobilized on a microscope slide and can be permeabilized to facilitate antibody access across the cell envelope. Indirect

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immuno-labeling requires the addition of a secondary antibody, conjugated to a fluorophore, while direct immuno-labeling omits the secondary antibody and conjugates the fluorophore to the primary antibody. Indirect IFM achieves a much higher fluorescent readout due to amplification of the signal with multiple fluorescently conjugated secondary antibodies.

Using IFM, we present here a protocol to localize a lipoprotein that contains a membrane-bound lysozyme inhibitor of c-type lysozyme (MliC) domain in *Fusobacterium nucleatum* – a Gram-negative oral anaerobe that is associated with oral disease, preterm birth, and colorectal cancer [3]. A highly conserved and ancient constituent of the innate immune system of mammals, lysozyme protects hosts from pathogenic bacteria by hydrolyzing peptidoglycan. Unfortunately, bacteria have evolved to counter the activity of cell wall hydrolase enzymes via the production of lysozyme inhibitors. First identified as Ivy (Inhibitor of vertebrate lysozyme) in *E. coli* [4] and later renamed as MliC [5], MliC proteins have been identified in many other bacteria, including *Pseudomonas aeruginosa* and *Salmonella* Typhi [5]. MliC proteins are predicted to anchor to the periplasmic side of the outer membrane [6, 7]. In *F. nucleatum*, MliC is a lipoprotein that harbors a lipobox [8], presumably promoting the membrane localization of this protein. Using *F. nucleatum* MliC as an experimental model, we provide a detailed procedure to visualize protein dynamics within the bacterial cell envelope. This procedure can be used for protein visualization in other Gram-negative bacteria.

2 Materials

All solutions should be prepared with sterile molecular grade water.

2.1 Coverslip Preparation

1. No. 1 coverslip (12 mm diameter).
2. 0.01% poly-L-lysine solution: prepare this solution from lyophilized poly-L-lysine hydrobromide in water.

2.2 Preparation of F. nucleatum Cells for Fixation

1. *Fusobacterium nucleatum* strains: Δ*mliC*, Δ*mliC*/pMliC, and CW1 (parent) [9].
2. TSPC: Tryptic Soy Broth (TSB) supplemented with 1% Bacto peptone and 0.25% freshly made L-cysteine.
3. 1.5 mL Eppendorf tubes.
4. Phosphate buffered saline (PBS), pH 7.4.
5. 4% formaldehyde in PBS.

2.3 Permeabilization of Bacterial Cells

1. 70% ethanol.
2. TEG Buffer: 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose.
3. 50 μg/mL lysozyme solution in TEG buffer.
4. End-to-end rotator.

2.4 Immunolabelling of Cells

1. 3% bovine serum albumin (BSA) solution.
2. Primary antibodies: rabbit anti-MliC (α-MliC).
3. Secondary antibodies: goat anti-rabbit IgG AlexaFluor 488 conjugate.

2.5 Mounting and Fluorescence Microscopy

1. Microscope slides (25 mm × 75 mm × 1 mm).
2. Anti-fade Mounting Medium with DAPI.
3. Hanks' Balanced Salt Solution (HBSS): 1.67 mM $CaCl_2.2H_2O$, 0.81 mM $MgSO_4$, 5.37 mM KCl, 0.44 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 0.27 M NaCl, 0.34 mM Na_2HPO_4, 5.55 mM D-glucose, pH 7.4.
4. FM™ 4–64 Dye: 500 μg/mL in HBSS.
5. Nail polish.
6. Dumont angled tweezers.
7. Fluorescence microscope with appropriate fluorescence filters.

3 Methods

3.1 Coverslip Preparation

1. Coat one side of coverslips with 150 μL of poly-L-lysine solution and let settle for at least 5 min (*see* **Note 1**).
2. Aspirate poly-L-lysine solution off coverslips and thoroughly rinse the coated surface with sterile molecular grade water.
3. Allow to air-dry for at least 2 h.

3.2 Preparation of F. nucleatum Cells for Fixation

1. Aseptically transfer a single colony of individual *F. nucleatum* strains to 5 mL of TSPC and grow the cultures at 37 °C in an anaerobic chamber.
2. Adjust the cell cultures to OD_{600} of 1.0 in 1.5 mL Eppendorf tubes and pellet the cells by centrifugation in a microfuge at 6000 × *g* for 2 min.
3. Wash the cell pellets twice in PBS and discard the supernatants.
4. For detection of non-surface proteins, proceed to Subheading 3.3.
5. For detection of outer membrane surface proteins, resuspend the cell pellets in 1 mL of PBS, transfer 150 μL of cell suspension to individual coated coverslips (*see* Subheading 3.1),

incubate at room temperature for 20 min, and aspirate the cell suspension.

6. To fix cells, apply 150 µL of 4% formaldehyde solution (*see* **Note 2**) onto coverslips and incubate at room temperature for 20 min.
7. Wash coverslips once with 150 µL of PBS (*see* **Note 3**) and aspirate PBS.
8. Proceed to Subheading 3.4.

3.3 Permeabilization of Bacterial Cells

1. Resuspend the pellets prepared from **step 3** of Subheading 3.2 in 1 mL of 4% formaldehyde and incubate at room temperature for 30 min.
2. Pellet cells by centrifugation at 6000 × *g* for 2 min and wash cell pellets twice in 1 mL of PBS.
3. Discard the supernatants, resuspend the cell pellets in 1 mL of 70% ethanol, and incubate at room temperature for 1 h on an end-to-end rotator.
4. Harvest the cells by centrifugation at 6000 × *g* for 2 min and discard the supernatants.
5. Resuspend the pellets in 0.5 mL of TEG Buffer, add 0.5 mL of 50 µg/mL lysozyme solution in TEG, and incubate at room temperature for 30 min.
6. Transfer 150 µL of the cell suspension above to the coated coverslips (Subheading 3.1) and incubate at room temperature for 5 min.
7. Carefully aspirate the remaining solution from the coverslips and wash cells bound to the coverslips with 150 µL of PBS.
8. Proceed to Subheading 3.4.

3.4 Immunolabeling of Cells

1. Block cells on the coverslips with 150 µL of 3% BSA solution at room temperature for at least 1 h and aspirate the solution.
2. Dilute the primary antibody in 3% BSA (1:200 dilution) and add 150 µL of the primary antibody solution onto coverslips and incubate at room temperature for 1 h.
3. Wash coverslips three times with PBS (*see* **Note 3**).
4. Dilute the secondary antibody in 3% BSA and add 150 µL of the secondary antibody solution onto coverslip and incubate at room temperature for 1 h *in the dark* for remaining steps to prevent photobleaching (*see* **Note 4**).
5. Wash coverslips three times with 150 µL of PBS.

3.5 Mounting and Fluorescence Microscopy

1. Add one drop of Anti-fade Mounting Medium with DAPI onto a microscope slide.
2. To visualize bacterial membranes, add 1–2 μL of FM 4-64 dye (500 μg/mL) onto the microscope slide and mix with DAPI above.
3. Using Dumont angled tweezers, invert coverslip and mount face down onto the microscope slide, allowing to sit for 1 min in the dark.
4. Carefully apply nail polish along the perimeter of the coverslips to seal and dry for 5 min (*see* **Note 5**).
5. Image via a fluorescence microscope, using the appropriate channel for the indicated excitation and emission spectra (*see* **Note 6**).

4 Notes

1. May incubate coverslips with poly-L-lysine solution overnight.
2. Store 4% formaldehyde solution at 4 °C.
3. For each wash, gently pipette PBS up and down near the edge of coverslips and leave PBS on coverslips for at least 5 min prior to aspiration.
4. Use a small box to cover coverslips as to keep them in dark. Avoid exposing coverslips containing fluorescently labeled secondary antibodies to light.
5. Prepared glass slides can be stored at −20 °C for future imaging; however, moisture at −20 °C may lessen the integrity of the seal. If reimaging, one may need to apply nail polish to reseal.
6. For AlexaFluor 488, use the green fluorescence channel (max excitation and emission at 490 nm and 525 nm, respectively). For DAPI, use the blue fluorescence channel (max excitation and emission at 350 nm and 470 nm, respectively). For the FM 4-64 dye, use the red fluorescence channel (max excitation and emission at 515 nm and 640 nm, respectively) (Fig. 1).

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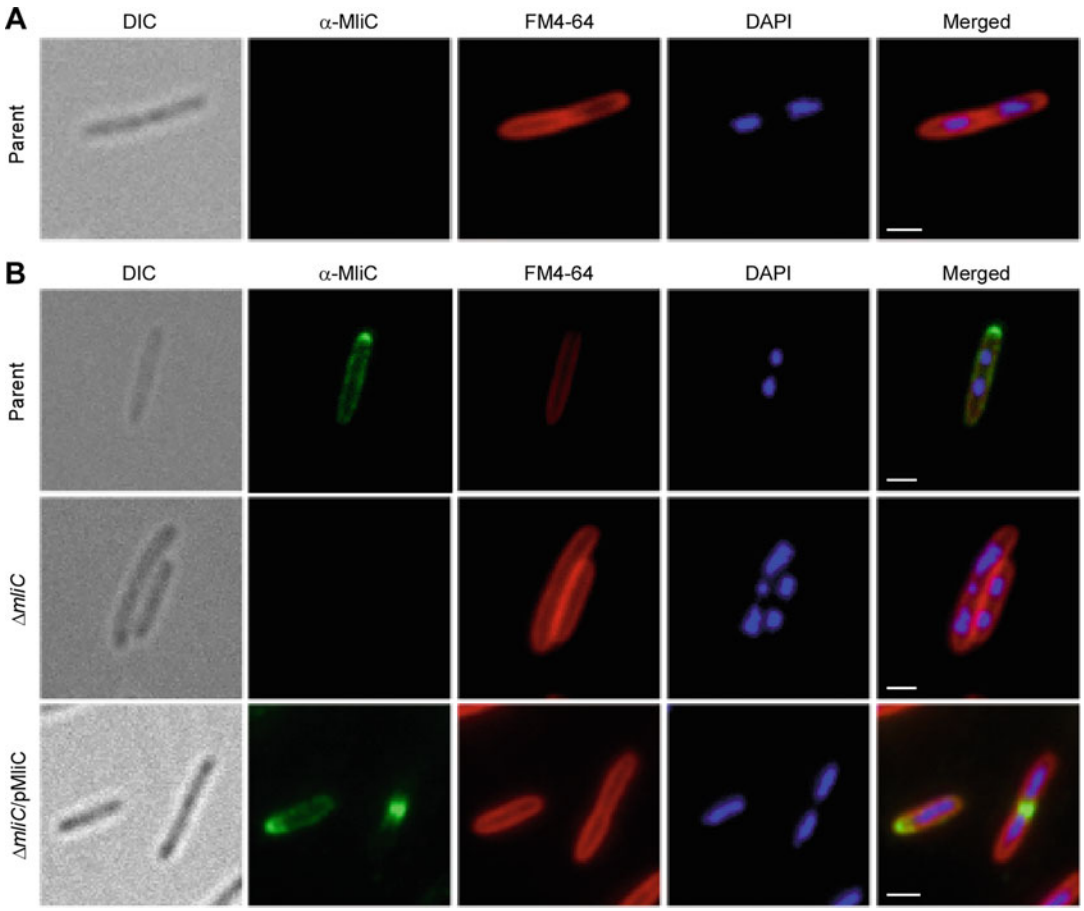

Fig. 1 Immunofluorescent microscopy of MliC. (**a**) Non-permeabilized cells of the parent strain and (**b**) permeabilized cells of the parent, Δ*mliC*, and Δ*mliC*/pMliC strains were stained with α-MliC (green), FM 4-64 (red), and DAPI (blue). Fluorescence signal was analyzed by a fluorescence microscope equipped with appropriate filters. Overlay of fluorescent images is shown (merged), with scale bars of 1 μm. Note, no MliC signal was detected in non-permeabilized cells, indicating MliC is not displayed on the extracellular side of the outer membrane

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Chapter 4

Computational and Biophysical Approaches to Identify Cell Wall-Associated Modulators in *Salmonella enterica* serovar Typhi

Mukesh Kumar, Md. Anzarul Haque, and Punit Kaur

Abstract

An increase in the number of antibiotic-resistant bacterial pathogens, in recent times, has posed a great challenge for treating the affected patients. This has paved the way for the development and design of antibiotics against the previously less explored newer targets. Among these, peptidoglycan (PG) biosynthesis serves as a promising target for the design and development of novel drugs. The peptidoglycan cell wall synthesis in bacteria is essential for its viability. The enzyme class, Mur ligases, plays a key role in PG biosynthesis. Therefore, compounds with the ability to inhibit these enzymes (Mur ligase) can serve as potential candidates for developing small modulators. The enzyme, UDP-N-acetyl pyruvyl-glucosamine reductase (MurB), is essential for PG biosynthesis, a crucial part of the bacterial cell wall. The development of novel drugs to treat infections may thus focus on inhibiting MurB function. Understanding the mechanism of action of Mur B is central to developing efficient inhibitors. For the treatment of S. typhi infections, it is also critical to find therapeutic drugs that specifically target MurB. The enzyme Mur B from *Salmonella enterica serovar* Typhi (stMurB) was expressed and purified for biophysical characterization to gauge the molecular interactions and estimate thermodynamic stability, for determining attributes for possible therapeutic intervention. The thermal melting profile of MurB was monitored by circular dichroism (CD) and validated by performing differential scanning calorimetry (DSC). An in silico virtual screening of various natural inhibitors was conducted with modelled stMurB structure. The three top hits (quercetin, berberine, and scopoletin) obtained from in silico screening were validated for complex stability through molecular dynamics (MD) simulation. Further, fluorescence binding studies were undertaken for the selected natural inhibitors with stMurB alone and with its NADPH-bound form. The natural inhibitors, scopoletin and berberine, displayed lesser binding to stMurB compared to quercetin. Also, a stronger binding affinity was exhibited between quercetin and stMurB compared to NADPH and stMurB. Based on the above two findings, quercetin can be developed as an inhibitor of stMurB enzyme.

Key words Molecular modeling, Molecular docking, Molecular dynamics simulation, Fluorescence binding, Differential scanning calorimetry, Natural compound Inhibitors, Mur ligase

Authors Mukesh Kumar, Md. Anzarul Haque have equally contributed to this chapter.

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1 Introduction

Bacterial pathogens developing resistance to multiple antibiotics simultaneously constitute a widespread burden in recent times. This "antibiotic resistance crisis" if not resolved in the near future can create a situation where treatment of infections that are deemed curable today may not be easily attainable. It is, therefore, necessary to design novel antimicrobial strategies to attenuate this situation. The first step toward achieving this is to search for or design a novel class of drugs that can escape bacterial resistance mechanisms. Antibacterial drugs are developed based on two approaches, either by targeting whole live multiplying cells (i.e., to stop cell division) or by targeting essential bacterial enzymes needed for cell viability. In the second approach, the early steps involved in the biosynthesis of bacterial cell wall (peptidoglycan(PG) component) are among the most imperative and underutilized therapeutic targets in drug discovery [1]. The enzymes involved in the PG biosynthesis pathway, Mur ligase, can prove to be a rescue mechanism from the "antibiotic resistance crisis" [2]. PG forms a necessary constituent of both Gram-positive and Gram-negative bacterial cell walls. The role of PG in bacterial cells is to maintain the osmotic pressure and thus, help bacteria to survive [3, 4]. The synthesis of PG is catalyzed by enzymes, Mur ligases A-F. These Mur ligases form attractive drug targets as these enzymes are not a part of the human enzyme pool. Hence, developing and designing new broad-spectrum therapeutics which inhibit these Mur ligases will lead to lesser side effects in humans [5]. Though numerous reports exist on the availability of inhibitors for several Mur ligases (including MurB), however, currently, compounds which demonstrate significant inhibition are not available [6–10]. Therefore, there exists a pressing need to develop novel antibacterial agents against the MurB enzyme to overcome the hurdles of drug-resistant pathogens.

Biological activity and stability of enzymes is determined by their specific three-dimensional native conformation [11]. Diverse covalent and non-covalent interactions maintain the enzymes in their functionally active stable state. Heat and certain chemical compounds (e.g., urea, GdmCl) induce changes in these interactions (both covalent and non-covalent) which leads to loss of biological function accompanied by partial or complete loss of native conformation [12]. Therefore, understanding the thermodynamics of protein structure, function, and stability will extensively help to develop and design precise inhibitors [13]. An in-depth recognition of the protein structural architecture requires detailed knowledge of the pathways along which the globular proteins fold. Thus, an analysis of protein thermal and chemical mediated folding/unfolding equilibrium is important for understanding the molecular basis of structure-activity relationships

[14]. This study was undertaken to observe the stability of enzyme MurB in the presence of heat and chemical (GdmCl and urea) denaturants. Hence, the protein MurB from *S.* Typhi (stMurB) was first cloned, expressed, and purified. Subsequently, the enzyme was subjected to heat-induced and chemical-induced denaturation process, to evaluate the structural changes induced by the denaturants on stMurB. The heat-induced denaturation profile of stMurB was obtained using CD spectroscopy and was validated by differential scanning calorimetry (DSC). Chemical-induced denaturation profile of the protein was studied using CD and fluorescence spectroscopy [15, 16]. Thermodynamic parameters T_m (midpoint of heat-induced denaturation) and H_m (enthalpy at T_m) obtained for protein were used to measure Gibbs free energy of protein [17, 18].

Likewise, it is also critical to find therapeutic drugs that specifically target stMurB. In the development of therapeutic agents against stMurB, several approaches can be employed. One approach is to design small molecule inhibitors that can specifically bind to the active site of the enzyme and inhibit its activity. Virtual screening, a computational approach, can be used to screen large databases of compounds to identify potential inhibitors. The identified compounds can then be further optimized using structure-based drug design techniques.

Computational approaches are important for the interaction of drugs with target molecules. A computational technique called homology modeling is used to predict the three-dimensional structure of a protein based on its amino acid sequence and the already-known structures of related proteins. Computational techniques like homology modeling, molecular docking, and Molecular Dynamics simulations are effective methods for finding cell wall-associated modulators. These techniques help scientists to create more potent antibacterial medications by helping them to comprehend the relationship between proteins and possible modulators.

Thus, a mix of in silico and in vitro techniques were undertaken to identify and validate potential natural compounds as inhibitors of stMurB.

2 Materials

2.1 Expression and Purification

1. *E. coli* BL21 (DE3) with pET28a harboring *stMurB* gene [19].
2. LB broth.
3. Antibiotics (kanamycin).
4. 1 M IPTG.
5. Floor centrifuge.
6. Sonicator (e.g., Q125 sonicator, Q SONICA).

7. Lysis buffer: 20 mM Tris, 500 mM NaCl pH 8.0 and 100 mM PMSF.
8. Wash buffer: 25 mM Tris, 150 mM NaCl, 50 mM imidazole, pH 7.4.
9. Elution buffer: 25 mM Tris, 150 mM NaCl, 150 mM imidazole, pH 7.4.
10. Ni-NTA resin.
11. HEPES buffer: 20 mM HEPES, 100 mM NaCl, and pH 7.4.
12. 30-kD Amicon filters.
13. Superdex 200 HiLoad 26/600 column.
14. 14% SDS-PAGE gels.

2.2 Characterization of Spectral Properties of Recombinant stMurB

1. Jasco spectropolarimeter (J-1500).
2. 0.1 cm cuvettes (cell).
3. 1 cm cuvettes (cell).
4. Purified stMurB protein (10 μM).

2.3 Temperature-Induced Denaturation

1. Jasco spectropolarimeter (J-1500) equipped with a Peltier-type temperature controller (ETCS-761).
2. 0.1 cm cuvettes (cell).
3. VP-DSC (GE Healthcare).
4. Purified stMurB protein (1.5 mg/ml).

2.4 Chemical-Induced Denaturation

1. Jasco spectropolarimeter (J-1500) equipped with a Peltier-type temperature controller (ETCS-761).
2. A quartz cuvette of 1 mm path length.
3. 1 cm cuvettes (cell).
4. Purified stMurB protein (5 and 10 μM).
5. 6 M GdmCl: Weigh 28 g GdmCl and dissolve in 30 ml Tris buffer. Finally set the volume to 50 ml by adding water.
6. 8 M Urea: Dissolve 24 g urea in 40 ml Tris buffer and finally set the volume to 50 ml by adding water.

2.5 Fluorescence Binding

1. Spectramax i3x.
2. 10 mM stock solutions of hit compounds (*see* **Note 11**).

3 Methods

3.1 Expression and Purification of Salmonella *Mur Ligase B (stMurB)* [19]

1. Grow *E. coli* BL21 (DE3) with pET28a harboring *stMurB* gene in 1 L of LB broth supplemented with 50 mM kanamycin at 37 °C till OD_{600} reaches 0.4–0.6.

2. Induce stMurB expression by adding 1 ml of 1 M IPTG (final concentration of IPTG-1 mM) to the above culture, grow the cells overnight at 20 °C, harvest cell by centrifugation at 6000× *g* in a floor centrifuge, and resuspend in 20 g cell pellets in 10 ml lysis buffer.
3. Lyse cells by ultra-sonication using Q125 sonicator (Q SONICA) on ice keeping the amplitude at 30% with on/off cycle at 30/45 s for 20 min.
4. Centrifuge lysed samples at 15000 *g* at 4 °C for 30 min to obtain the supernatant and mix the supernatant with 2 ml pre-equilibrated Ni-NTA resin and incubate for batch binding at 4 °C for 1 h.
5. Wash column with 5 times the column volume (CV) ml wash buffer.
6. Elute bound proteins with 2CV elution buffer.
7. Concentrate eluted fractions by spinning in Amicon filter of desired molecular weight cut off.
8. Perform size-exclusion chromatography using Superdex 200 HiLoad 26/600 column pre-equilibrated with HEPES buffer. To equilibrate column, flow 1 column volume of HEPES buffer (*see* **Note 1**).
9. Confirm the purity of purified proteins by SDS-PAGE electrophoresis with 14% SDS-PAGE gels and store purified proteins at −80 °C in 1 ml aliquots (*see* **Note 2**).

3.2 Characterization of the Spectral Properties of Recombinant stMurB

Far-UV circular dichroism (CD) and near–UV CD spectroscopy can be used to determine spectral properties of recombinant stMurB at pH 7.4 and 25 °C.

1. Turn on the computer system connected to the Jasco spectropolarimeter (*see* **Notes 3** and **4**).
2. Open the N_2 gas flow into the Jasco spectrophotometer and ensure the meter ball monitoring the flow of N_2 gas is above 40 always. Allow the N_2 gas to purge the compartments for at least 5 min (*see* **Note 5**).
3. Turn on the Jasco spectrophotometer. If the study requires the use of Peltier temperature controller, then water bath should be turned on and set at 20 °C.
4. Open the Spectra Manager Program and choose the program and set the required parameters, for the required experiment (*see* **Note 6**).
5. Take 0.1 cm path length cuvette (cell) for far-UV (250 – 200 nm) and 1.0 cm cuvette (cell) for near-UV (300–270 nm) CD spectral measurements.

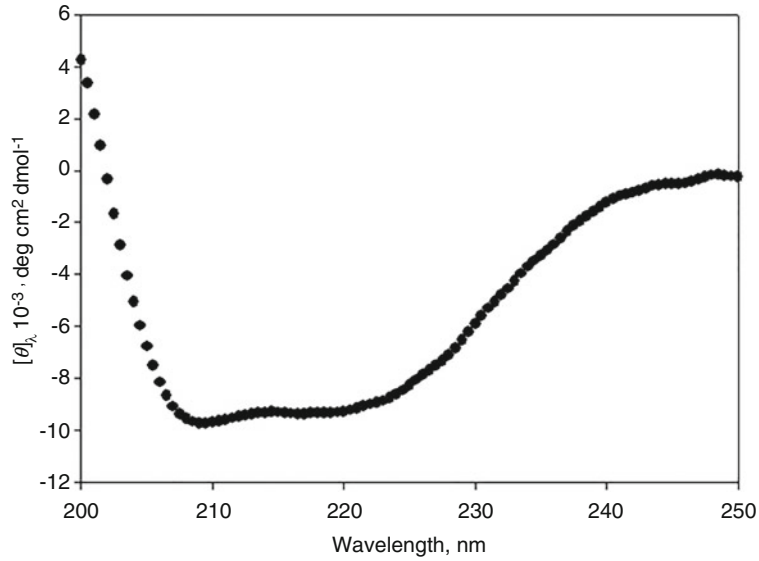

Fig. 1 Representative Far-UV CD spectra of protein taken at pH 7.4 and 25 ± 0.1 °C

6. Fill cuvette with protein sample (having constant protein concentration of 10 μM) prepared for experiment. Open the lid of Jasco spectropolarimeter and place the cuvette in cuvette holder (*see* **Note 7**).
7. Set the wavelength range and temperature click on "Run".
8. Correct each spectrum for contribution of the blank solution.
9. Start the scan (*see* **Note 8**).
10. Convert CD data to concentration-independent parameter (Fig. 1), the mean residue ellipticity $[\theta]$ (deg cm ^{2}dmol $^{-1}$), using the relation,

$$[\theta]\,\lambda = \mathrm{M_o}\,\theta_\lambda / 10lc \qquad (1)$$

where θ_λ is the observed ellipticity in milli degrees at wavelength λ, $\mathrm{M_o}$ is the mean residue weight of the protein, c is the protein concentration in mg ml^{-1}, and l is the path length of the cell in centimeters.

11. Calculate secondary structure content from far-UV CD spectra using $\mathrm{K_2D}$ program on the Dichroweb server (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) [20, 21].

3.3 Temperature-Induced Denaturation

Temperature-induced denaturation of stMurB can be performed with CD at 216 nm using Jasco spectropolarimeter (J-1500) equipped with a Peltier-type temperature controller (ETCS-761). To operate this CD machine for thermal denaturation, follow the same procedure as mentioned in Subheading 3.2 with some modifications. This can be done for all protein samples.

1. Fill the sample cell of 0.1 cm path length with 200 μl of protein (10 μM).
2. Set fixed wavelength of 216 nm and heat protein sample from 20 °C to 70 °C (Heating rate 1 °C/min).
3. Perform all the temperature scans in triplicate and take the average value of all three transition curves to prepare a plot.
4. Convert the raw CD data (θ_{216}, milli degree) into concentration-independent parameter, mean residue weight (MRE) ($[\theta]_{216}$, deg. cm^2 dmol^{-1}) using eq. (1).
5. Use non-linear least-squares analysis to analyze the transition curve for T_m (midpoint of denaturation) and ΔH_m (enthalpy change at T_m) according to the relation:

$$y(T) = \frac{\mathrm{yN\,(T) + yD(T)\ exp\ [-\Delta}Hm/\mathrm{R}(1/\mathrm{T} - 1/T\mathrm{m})]}{1 + \exp[-\Delta Hm/\mathrm{R}(1/\mathrm{T} - 1/T\mathrm{m})]} \quad (2)$$

where y(T) is the optical property at temperature T(K), y_N(T) and y_D(T) are the optical properties of the native and denatured protein at temperature T (K), and R is the gas constant.

6. Measure differential scanning calorimetry (DSC) with VP-DSC (GE Healthcare) using 1.5 mg/ml stMurB concentration in the temperature range of 20-70 °C.
7. Analyze DSC endotherm obtained by a simple two-state model provided with DSC instrument to estimate T_m, ΔH_m, and ΔC_p associated with structural change due to heating of stMurB at constant pressure (Fig. 2).

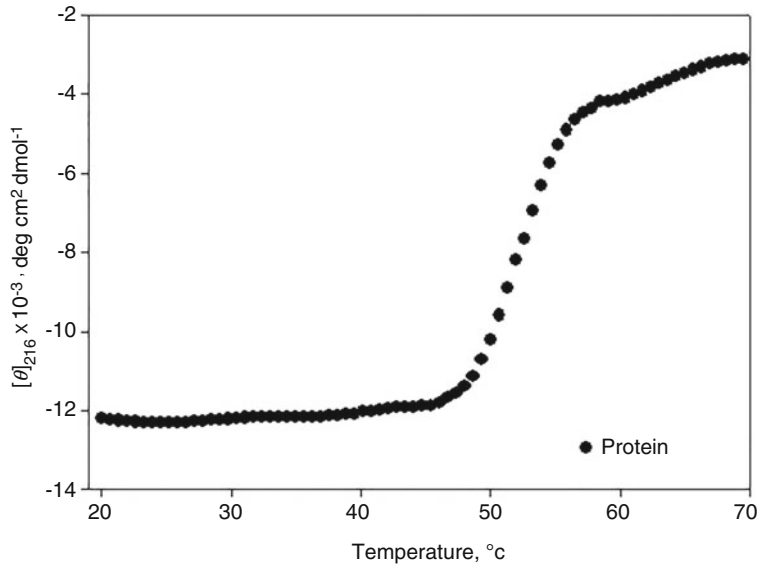

Fig. 2 Thermal denaturation of protein monitored by $[\theta]_{216}$ at pH 7.4

8. Use the values of T_m, ΔH_m, and ΔC_p (from DSC measurement) obtained to calculate the Gibbs free energy change (ΔG_D) at T (25 °C), using the Gibbs-Helmholtz equation (*see* **Note 9**).

$$\Delta G_D\ (T) = \Delta H_m \left(\frac{T_m - T}{T_m}\right) - \Delta C_p \left[(T_m - T) + Tln\left(\frac{T}{T_m}\right)\right] \quad (3)$$

3.4 Chemical-Induced Denaturation

The effect of denaturants (GdmCl and urea) on secondary structure and tertiary structure of protein can be monitored by Far-UV CD and fluorescence measurements, respectively.

1. Far UV-CD spectra measurements: Use 10 μM protein concentrations and a quartz cuvette of 1 mm path length.
2. Carry out baseline correction with the buffer.
3. Fluorescent spectra: For fluorescence spectra measurement, use 5 μM of proteins and 1 cm path length (*see* **Note 10**).
4. Normalize data to MRE ($[\theta]_\lambda$) in deg. cm^2 dmol^{-1} unit, using eq. (1). Check reversibility of protein diluting the chemically denatured protein with buffer [22, 23].

3.5 Fluorescence Binding

The fluorescence binding of hit compounds with stMurB protein can be examined by monitoring changes in fluorescence intensity using Spectramax i3x in the wavelength range of 300-400 nm at pH 7.4 and 25 °C.

1. Make 10 mM stock solutions (*see* **Note 11**).
2. Set up the excitation wavelength at 280 nm and the emission wavelength at 300-400 nm using Spectramax i3x.
3. Perform each experiment in triplicate, with blank subtracted to obtain the final spectra.
4. Plot and analyze changes in fluorescence intensity at λ_{max} (F_0-F) versus concentration of compounds in μM by one set of the binding model in SigmaPlot and using modified Stern-Volmer equation for the estimation of binding constant (K_a), number of binding sites (n),

$$\log \frac{Fo - F}{F} = \log \mathrm{Ka} + n \log[L] \quad (4)$$

where F_o is fluorescence intensity without ligand; F, the fluorescence intensity of protein in the presence of ligand; K_a, binding constant; n, number of binding sites; [L], ligand concentration.

3.6 In Silico Analysis

Computational approaches for identifying cell wall-associated modulators in Gram-negative bacteria typically involve a combination of several methods, out of which structure-based approaches have been used by our lab to identify novel small molecules. This structure-based approach relies on the availability of high-resolution structural information about the target protein. Some of the commonly used methods in this category include the following.

3.6.1 Homology Modeling

This involves building a 3D model of the target protein using the known structure of homologous proteins. Homology modeling can be used to predict the binding site of potential inhibitors and to perform virtual screening of compound libraries. It is a computational method used to predict the three-dimensional structure of a protein of interest based on its amino acid sequence and the known structure of a related protein or a homologous template. This technique relies on the observation that the amino acid sequence of a protein is more conserved than its three-dimensional structure. Thus, if two proteins have a sequence identity (usually more than 30%), they are likely to have a similar structure [24, 25].

The homology modeling process involves several steps, including template selection, alignment of the target sequence with the template, model building, and model refinement (*see* **Note 12**).

1. Access the Swiss-Model website, https://swiss-model.expasy.org/, and create an account, if necessary (*see* **Note 13**).
2. Enter the amino acid sequence of stMurB retrieved from the UniProt database; make sure the sequence is in FASTA format (*see* **Note 14**).
3. Select a suitable template PDB structure (e.g., *E. coli* MurB) from the Protein database (www.rcsb.org) that will be used for modeling. The template chosen from the PDB database should have a high degree of sequence similarity to the protein of interest (*see* **Note 15**).
4. Provide the sequence and template selection to the web-based program Swiss-Model and wait for the modeling procedure to complete. Depending on how large the protein sequence is and how complicated the model is, this could take several minutes or longer.
5. The model's quality and its structural closeness to the template structure are of utmost importance, hence evaluate generated model by superimposing the modelled structure (stMurB) onto the template structure (*E. coli* MurB; PDB:2MBR) in PyMol structure visualization software and calculate the root-mean-square deviation (RMSD).

6. Verify the generated model from Swiss-Model by several different programs to check its stereochemical quality. For example, PROCHECK's Ramachandran plot and G-factor were used. ProSA Z-score (*see* **Note 16**), ProSA Local energy profile (*see* **Note 17**), ProSA Verify 3D, and the ERRAT Plot (*see* **Note 18**) [26–28].
7. Use energy minimization and molecular dynamics simulations to fine-tune the model.
8. Compare the generated model to accessible experimental data, such as X-ray crystallography or nuclear magnetic resonance data, to ensure its accuracy. Use the homology model to proceed with experiments like ligand binding assays and protein-protein interaction investigations (*see* **Note 19**).

3.6.2 Molecular Docking

Molecular docking is a computational technique used to predict the binding mode and binding affinity of a small molecule ligand to a target macromolecule such as a protein or a nucleic acid. The ligand and target are modelled as flexible entities, and their possible binding conformations and orientations are explored using various algorithms and scoring functions. Glide in Maestro Schrödinger suite version 2020 [29, 30] is our preferred molecular docking software since it is fast, accurate, flexible, and customizable, despite the fact that other programs like Gold, Autodock have their own benefits.

The process of molecular docking in Glide Maestro typically involves the following steps:

1. To get started, open the Schrödinger suite of applications and go to the Maestro tab.
2. Prepare the refined and validated model structure from Homology modeling step for molecular docking. Add hydrogen atoms, assign bond ordering, and remove any excess ligands or water molecules in protein preparation wizard (*see* **Note 20**).
3. Put the ligand structure (e.g., EEB1) together by assigning bond ordering and adding hydrogen atoms. Create tautomers, ionization states, and low-energy conformations of the ligand with the help of the LigPrep module (*see* **Note 21**).
4. Open Maestro and import protein structure into the Workspace.
5. Launch SiteMap from the SiteMap panel in Maestro. Do this by clicking on "SiteMap" in the panel or by selecting "SiteMap" from the "Calculate" menu to predict the binding site (*see* **Note 22**).
6. In the SiteMap panel, select your protein structure from the drop-down menu under "Input Structure".

7. Click on the "Run SiteMap" button to start the analysis. SiteMap will analyze the protein structure and identify potential binding sites.
8. Once the analysis of the protein structure is complete, results will be displayed in the SiteMap panel. The potential binding sites will be displayed as colored spheres on the protein structure.
9. In the Receptor Grid generation tab choose the binding location for which a receptor grid should be constructed. In the SiteMap window, choose the matching-colored sphere to accomplish this.
10. Open the Receptor Grid Generator panel in Maestro to generate a receptor grid for a specific binding site. Using this grid generation module, grids of varying sizes (5-15 Å) can be generated around the centroid of the bound ligand EEB1 to represent the cube where the ligand is predicted to bind (*see* **Note 23**).
11. Load the prepared Receptor Grid protein structure and the ligand into the Glide module and set the docking parameters to high-throughput virtual screening (HTVS), standard precision (SP), or Extra Precision Docking (XP). Choose the docking algorithm and the scoring function according to our objective (*see* **Note 24**).
12. Consider the outcomes of the docking simulation. The docking score, binding mode, and ligand-protein interactions are all important considerations.
13. Adopt Post-docking minimization and in-depth visual inspection of the binding location to fine-tune the docking results (*see* **Notes 23** and **24**).
14. Select the top docked complex for MD simulations study for time scale of 100 ns using Desmond suite to gain insight into the overall stability of the complex as well as local and global conformational fluctuations in protein and ligand atoms in an explicit water model.

3.6.3 Molecular Dynamics Simulations

Proteins are inherently dynamic, making molecular and atomic-level analyses of their motions crucial to deciphering key physicochemical behaviors. This method involves simulating the dynamic behavior of the target protein and its interactions with potential inhibitors. Molecular dynamics simulations can be used to predict the stability of protein-inhibitor complexes (docked complex), and to identify potential hotspots for inhibitor binding. The MD simulation study facilitated the understanding of the structural and conformation behavior of docked complex in physiological solvent system. We used the Desmond module of Schrödinger suite version 2020 for MD simulation study [31]. The procedure for doing

a molecular dynamics simulations in Desmond is as follows (*see* **Note 25**):

1. Start by going to the Desmond software page in the Maestro interface of the Schrödinger suite.
2. Get the selected docked complex ready for MD simulations by including hydrogen atoms, assigning bond ordering, and reducing the system's energy.
3. Position the docked complex initially using the system builder module in an orthorhombic box with periodic boundary conditions that had borders that were at least 10 Å × 10 Å × 10 Å away from any neighboring solutes or atoms across the protein structure on all sides. This tool creates an explicit solvated system model SPC, and in order to simulate physiological conditions, neutralize the system by adding 0.15 M counter ions (NaCl) to the solvent box.
4. Using Desmond's minimization tool, energy minimize the system using default settings to equilibrate and lower the system's overall potential energy. For all MD simulations, set relaxation time to 1 ps, and use OPLS2005 force field parameters for each complex. Choose the particle mesh EWALD approach to determine the complex's long-range electrostatic interactions.
5. Use isothermal isobaric ensemble (NPT) at a constant temperature of 300 K and pressure of 1.013 bar for each docked complex with a production run of 100 ns. Remember to save each resultant trajectories of the simulated systems at 100 ps intervals for later examination.
6. Perform an energy minimization phase to eliminate any unfavorable connections or steric conflicts inside the solvated system. The steepest descent algorithm is commonly used for a few hundred to several thousand steps, or until the energy converges.
7. Conduct the MD simulation and analyze the results, such as the protein-ligand interactions, system stability, and period of the system.
8. Calculate the root mean square deviation (RMSD, *see* **Note 26**) of the positions of the protein backbone atoms and the local protein regions with the highest fluctuations using root mean square fluctuation (RMSF, *see* **Note 27**) with time and analyze obtained trajectories using Desmond's Simulation Interaction Diagram (SID) tool to observe the stability of the protein-ligand complex.
9. Evaluate the trajectory and determine various parameters, including RMSD, RMSF, and binding free energy, and Simulation Interaction diagram, Simulation quality analysis, and Simulation event analysis tools, in Maestro.

4 Notes

1. For this step, a FPLC system of AKTA Pure (GE Healthcare Life Science) can be used.
2. Protein samples can be stored in aliquots so that only one aliquot can be used at a time to prevent rethawing and refreezing of sample as this can degrade the protein.
3. To obtain proper CD signal, CD machines should be regularly calibrated to set correct ellipticity and wavelength. With camphor-10-sulfonic acid (CSA) commonly used as calibration standard, CSA at 1 mg/ml in a 1 cm cell give the ellipticity ratio at 192.5 to 290.5 between 2.05 and 2.08.
4. Ozone produced by CD lamp will destroy mirror in the optics. Nitrogen must be flushed to remove oxygen before turning machine on to prevent ozone formation.
5. Purging of compartments with N_2 gas is done for two reasons: (i) to protect the optics from the harmful effects of ozone formed by Xe lamp used in the instrument, and (ii) ozone absorbs in the range of 200-300 nm and 600-700 nm which may interfere with our experimental study).
6. Cuvette should be clean and without mechanically strained as stain depolarizes light and CD baseline change.
7. Protein sample is prepared low absorbing buffer and highly pure (more than 95% protein should be the protein of interest).
8. Measure at least 5-7 accumulation of CD spectra of sample and baseline. Monitor HT signal and make sure it does not exceed the linear range of CD machine.
9. All the measurements would be assumed that ΔC_p is not dependent on temperature.
10. Use separate samples for each concentration of denaturant concentration (0-6.0 M for Urea and 0-4 M for GdmCl) for titration of protein with denaturant. Use stock solution of urea and GdmCl for titration experiment.
11. Weigh desired compounds and dissolve in DMSO. Make the final dilution of natural compounds working buffer (20 mM HEPES, 100 mM NaCl, and pH 7.4). Dissolve 0.476 g of HEPES and 0.584 g NaCl in 90 ml water, set pH 7.4 and finally make the volume 100 ml. Final DMSO <4% in working samples.
12. Due to lacking a crystal structure of stMurB, the three-dimensional (3D) model of stMurB was constructed by comparative homology modeling. The protein data bank (PDB) database was searched using the stMurB amino acid sequence retrieved from the UniProt database (Accession ID of

Q8Z316) in order to identify the best homolog based on a set of criteria, including the best sequence identity, high sequence similarity, an expectation value (E-value) of 0 indicating a highly significant match, and low-resolution value (<2.0 Å) of the resolution.

Template selection: At this stage, a template structure is chosen from the PDB database that is highly similar to the target protein in terms of sequence. The template's resolution should be high, and it should have a similar structure to the protein of interest [24].

Sequence alignment: The target protein's amino acid sequence must then be aligned with the template protein. For this, a variety of software packages including ClustalW, MAFFT, and T-Coffee are employed [32–34].

Model building: Once the sequences have been aligned, the target protein's structure can be constructed by referencing the coordinates of the template protein. MODELLER, SWISS-MODEL, and ROSETTA are just a few of the popular modeling software packages [35, 36].

Model refinement: Finally, the model is optimized and subjected to energy minimization to fix any lingering steric conflicts and enhance its overall quality. For model improvement, scientists employ a wide range of molecular mechanics force fields and optimization tools, including CHARMM, AMBER, and GROMOS [37–40].

13. When it comes to the minimum proportion of sequences needed for homology modeling with Swiss-Model, there is no hard and fast rule. For successful modeling, however, at least 30% sequence identity between the target protein and the template structure is often recommended. In general, a model's quality improves as the sequence identity value increases. In addition, when selecting a model to replicate, keep in mind how essential it is to maintain the original's structure and functionality.
14. The FASTA format is a standard format where the first line is the identifier and begins with > and includes notes regarding the sequence. The second and following lines are the sequence of the protein in "one letter code" for amino acid residues.
15. Sequence similarity between the two sequences is more than 80%.
16. https://prosa.services.came.sbg.ac.at/prosa.php
17. https://prosa.services.came.sbg.ac.at/prosa.php
18. https://saves.mbi.ucla.edu

19. Overall, homology modeling is a reliable strategy for predicting the structure of uncharacterized proteins, and it has numerous possible applications in areas such as drug discovery and protein engineering. The accuracy of the homology model, however, depends on two factors: the quality of the alignment and the degree of similarity between the target and template sequences.
20. *Protein preparation*: Protein preparation optimized the target protein's energy-minimized structure mode. Other hetero molecules like water were removed, hydrogen atoms were introduced, and geometric clashes were reduced. The prime suite introduced the side-chain residues that were absent, and the bond shape was adjusted. Using the optimal potentials for liquid simulation (OPLS)-2005 force field and default constraints of 0.30 Å for root mean square deviation (RMSD), protein-energy minimization was carried out with impref [40].
21. *Preparation of the ligand*: The 3D structures of the ligand and target molecules are obtained from experimental or computational sources and are optimized to remove any steric clashes or distorted bonds using molecular modeling software. Ligands were prepared by the Ligprep module with low energy states for further modeling studies [41]. Tautomeric and ionization states in pH range 7 ± 2.0 were created using EPIK [42] and the ligand sets geometric optimization was performed using the OPLS-2005 force field [43]. The lower energy stereoisomers produced for each ligand with accurate chiralities were considered. Ligprep generated a maximum of 32 confirmations for each structure, with optimized ionic states, tautomer, stereochemical and ring conformations. The software Schrödinger (Release 2020) and its protocols were utilized for this study.
22. *Generation of the receptor grid*: The region around the target macromolecule where the ligand can potentially bind, known as the search space, is defined using various criteria such as the active site, allosteric sites, or surface pockets. Small molecule docking requires first locating the binding site. Target enzyme binding site was identified using a manual correlation of the enzyme's amino acid sequence and data from the sitemap tool [44] in Maestro. The binding pocket is calculated by utilizing the interaction energies to find the most favorable locations energetically. Docking experiments necessitate the creation of a receptor grid, which specifies a cube in which the ligand is expected to bind. The site map program's projected binding site in the protein model structure was used to guide the construction of the receptor grid, which was built with radii between 20 Å.

23. *Virtual screening/docking*: The most effectively docked ligand molecules with the target enzyme were found via high-throughput virtual screening. Glide, made by Schrödinger Corporation, is a commercial docking application in which docking at the protein active site was performed utilizing the Glide virtual screening module [45–47]. In order to find the optimal shape, orientation, and position of a ligand in a certain receptor, the glide docking package exhaustively searches that space. The receptor grid was initially derived by projecting the center of the residues in the protein's active site outwards. There were three stages of filtering in the screening process, with particular cutoffs accordingly [48]. High-throughput virtual screening (HTVS), followed by standard precision (SP), and lastly, filter criteria from Extra Precision Docking (XP) [29]. HTVS and SP docking use the identical scoring function, but HTVS reduces the number of intermediate conformations in the docking funnel and the thoroughness of the final torsional refinement and sampling. Both are having same docking algorithm. XP samples more than SP before starting its anchor-and-grow procedure. XP has a more advanced scoring system that requires better ligand-receptor form complementarity than SP Glide Score. This eliminates SP false positives. Ideally, dock to various receptor conformations because XP penalizes ligands that don't fit well. XP docking is an approach that utilizes a novel scoring system and an improved sampling algorithm. Compared to previous scoring functions inside Glide, the technique has shown significant gains in binding affinity prediction and database enrichment when evaluated with a wide variety of ligands and receptors [29]. A glide scoring function, ligand efficiency, and glide energy were then used to evaluate the hits from the final screening step, and their interactions with the active site residues were analyzed.
24. *Docking validation and analysis*: The generated docking conformations are ranked according to the free energy of binding, which is calculated using a semi-empirical scoring function. The approach combines incremental construction for conformational sampling of the ligand with partial flexibility of the receptor. The top-ranked ligand poses are analyzed and validated using experimental or computational methods such as molecular dynamics simulations, free energy calculations, or binding assays. The docked ligand poses can be refined using optimization techniques such as induced fit docking or hybrid methods that combine docking with other computational techniques such as molecular dynamics simulations.

25. *Molecular dynamics simulations*: The procedure for doing a molecular dynamics simulations in Desmond is as follows:

System preparation: The molecular system must be prepared for simulation in which initially import a 3-D structure file into Maestro [48]. Next step is to prepare the protein structure file using protein preparation wizard in which we eliminate ions and molecules that are, for example, crystallization artifacts, set the proper bond orders, add hydrogens, detect disulfide bonds, and fill in any missing side chains or whole residues. Finally, make any necessary adjustments to the protonation and tautomerization of variable residues, as well as ligands and co-factors (if present). After the system preparation, we must configure the simulation's settings, such as the simulation's time, temperature, and pressure. System Builder tool can be used to design the simulation parameters. The system builder module was exercised to initially place the docked complex in an orthorhombic box with periodic boundary conditions with edges at least 10 Å × 10 Å × 10 Å away on all sides from any nearby solute or atoms across the protein structure. This tool generates an explicit solvated system model (SPC, TIP3P, TIP4P, TIP5P, etc.) and further, the system was neutralized by adding 0.15 M counter ions (NaCl) into the solvent box to mimic the physiological conditions.

Energy minimization: It is necessary to carry out energy minimization after the system has been configured in order to eliminate any steric conflicts between atoms and relax the system. To make sure the system is stable before the simulation itself, this is a crucial step. The Desmond Energy Minimization tool can be used to minimize by steepest descent and LBFGs algorithm with a maximum of 2000 iterations with convergence criteria of 1 kcal/mol/Å.

Equilibration and production run: The next step is to equilibrate the system in order to achieve the desired temperature and pressure. This step enables the system to adapt to the simulated conditions prior to the simulation itself. Equilibration is followed by the beginning of the production run that will execute the actual MD simulation. Here, classical mechanics is used to mimic the system's dynamics. To begin the simulation, we can utilize the Desmond Production Run tool. Finally, the production run of 50 ns was performed for all the energy minimized complexes in eight discrete phases with specific limitations. The initial seven phases of molecular dynamics included the equilibration process, while the last step constituted the extensive simulation production phase. The long-

lasting production phase was carried out for 50 ns at a constant temperature (300 K) using the Nose–Hoover chain coupling scheme. The reversible reference system propagator algorithms (RESPA) integrator was used throughout the molecular dynamics simulation. The bonding interactions for a time step of 2 fs were calculated in the final production run. The Particle Mesh Ewald (PME) algorithm was used to determine the long-series electrostatic interactions during the simulation [49–51].

Analysis: After the simulation is completed, we can examine the simulation trajectory to learn more about the system's behavior through time. After completion of the production run, simulation trajectories were analyzed by a variety of analytical tools, such as simulation event analysis, simulation interaction diagram, and simulation quality analysis, which enables us to examine characteristics like hydrogen bonding, root-mean-square deviation (RMSD), root mean square fluctuation (RMSF), and the number of hydrogen bonds formed during the simulation and other molecular interactions [50].

26. *RMSD*: The average change in displacement of a particular set of atoms for a given frame relative to a reference frame is calculated using the Root Mean Square Deviation (RMSD). It is determined for every frame of the trajectory. For small, globular proteins, changes of the order of 1–3 Å are quite acceptable. However, changes considerably larger than that suggest that the protein is going through a significant structural shift throughout the simulation. The RMSD of a ligand demonstrates its stability in relation to a protein and its binding pocket. If the reported values are significantly higher than the protein's RMSD, the ligand has undoubtedly pushed from its initial binding site.
27. *RMSF*: The Root Mean Square Fluctuation (RMSF) is useful for characterizing local changes along the protein chain. The protein's N- and C-terminal tails typically change more than any other region of it. Beta strands and alpha helices are examples of secondary structure components that are often more rigid than the protein's unstructured portion and exhibit less fluctuation than loop regions.

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Chapter 5

Employing Cloning-Independent Mutagenesis of *Parvimonas micra* for the Study of Cell Wall Biogenesis

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Abstract

The cell wall plays an important structural role for bacteria and is intimately tied to a variety of critical processes ranging from growth and differentiation to pathogenesis. Our understanding of cell wall biogenesis is primarily derived from a relatively small number of heavily studied model organisms. Consequently, these processes can only be inferred for the vast majority of prokaryotes, especially among groups of uncharacterized and/or genetically intractable organisms. Recently, we developed the first tractable genetic system for *Parvimonas micra*, which is a ubiquitous Gram-positive pathobiont of the human microbiome involved in numerous types of inflammatory infections as well as a variety of malignant tumors. *P. micra* is also the first, and currently only, member of the entire *Tissierellia* class of the *Bacillota* phylum in which targeted genetic manipulation has been demonstrated. Thus, it is now possible to study cell wall biogenesis mechanisms within a member of the *Tissierellia*, which may also reveal novel aspects of *P. micra* pathobiology. Herein, we describe a procedure for cloning-independent genetic manipulation of *P. micra*, including allelic replacement mutagenesis and genetic complementation. The described techniques are also similarly applicable for the study of other aspects of *P. micra* pathobiology and physiology.

Key words *Parvimonas micra*, Genetics, Peptidoglycan biosynthesis, Cell wall, Gram-positive anaerobe

1 Introduction

Parvimonas micra is a pathogen of the oral cavity as well as other extraoral sites in the human body. *P. micra* is a member of the *Tissierellia* class of the *Bacillota* phylum. Studies within *Tissierella* have been severely limited due to the lack of genetic tools, and as such, no genetic studies of cell wall biogenesis have been performed in any representatives of this class. Given the essential biological role of the bacterial cell wall, these studies will be critical to our understanding of the physiology and pathogenesis of these microbes [1–3].

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P. micra is included among the Gram-positive anaerobic cocci (GPAC) group, which accounts for the majority of anaerobic bacteria isolated from many types of clinical specimens [4]. It is most strongly associated with both chronic and acute inflammatory diseases of the human mucosae and has been proposed as a discriminating biomarker for multiple types of cancer [5]. Despite the mounting evidence for the central role of *P. micra* in the pathogenesis of numerous diseases, genetic studies are severely lacking. Accordingly, the mechanisms of *P. micra* cell wall biogenesis and its connection to disease have yet to be elucidated. However, the *P. micra* cell wall has been previously demonstrated to serve as a potent inflammatory mediator in human macrophages, eliciting the secretion of TNF-α, IL-1β, IL-6, IL-8, and RANTES [6]. In this chapter, we provide specific details of the steps required to perform cloning-independent genetic mutagenesis in *P. micra*, which can be employed for the study of cell wall biogenesis in this poorly characterized organism [7]. A better understanding of this process is likely to reveal new insights into *P. micra* physiology and pathobiology, while also providing new potential therapeutic targets to treat the plethora of *P. micra*-associated infections.

2 Materials

2.1 Bacteria Culture (see Note 1)

1. Freezer stocks (−80 °C) of *Parvimonas micra* bacteria in sBHI with 25% (v/v) glycerol in cryovials; wild-type reference strain ATCC 33270 [8] or other wild-type naturally competent strain [7] (*see* **Note 2**).
2. Syringe filters (0.22 μm) and compatible 10 mL syringe.
3. Antibiotic stock solutions: kanamycin 100 mg/mL (w/v) in double distilled water (ddH_2O), sterilized by passage through a syringe and 0.22 μm filter, and divided into aliquots. Erythromycin 25 mg/mL (w/v) in ethanol. Store aliquots of antibiotics at −20 °C until use.
4. Media supplements: hemin 10 mg/mL (w/v) in 0.1 M NaOH, menadione 10 mg/mL (w/v) in ethanol, L-cysteine hydrochloride monohydrate 50 mg/mL (w/v), yeast extract, and agar.
5. Supplemented brain heart infusion (sBHI) medium. Dissolve 37 g of brain heart infusion media (BD or equivalent) into 1 liter of distilled water with stirring. Add 5 g of yeast extract, 10 mL of L-cysteine solution, and 0.5 mL of hemin solution. Autoclave media for 15 min at 121 °C. Allow to cool to 50–55 °C in a water bath. Add 1 mL of menadione solution and mix.
6. Sterile Petri dishes (100 mm × 15 mm).

7. Supplemental brain heart infusion agar (sBHI agar). Dissolve 37 g of brain heart infusion media into 1 L of distilled water with stirring. Add 5 g of yeast extract, 10 mL of L-cysteine solution, 0.5 mL of hemin solution, and 15 g of agar with stirring. Autoclave media for 15 min at 121 °C. Allow to cool to 50–55 °C in a water bath. Add 1 mL of menadione solution and mix. For the selection and growth of mutant strains, add the appropriate amount of antibiotic stock solutions to achieve final antibiotic concentrations of 15 μg/mL (w/v) erythromycin or 300 μg/mL (w/v) kanamycin and mix gently. Dispense into Petri dishes and allow to solidify.
8. Disposable inoculating loops (1 μL).
9. Anaerobic chamber (85% N_2, 10% CO_2, 5% H_2) at 37 °C.

2.2 Mutagenesis Construct Assembly

1. PCR supplies: molecular biology grade double distilled water (ddH_2O), high fidelity polymerase (e.g., Phusion and corresponding buffers, Fisher Scientific), 10 mM dNTP stock, template DNA, forward and reverse primers (to be designed specifically for the target gene of interest), nested forward and reverse primers (to be designed specifically for the target gene of interest), thermocycler, and compatible PCR tubes.
2. Agarose gel electrophoresis supplies: TAE buffer (40 mM tris base, 20 mM acetic acid, 1 mM EDTA), 1.0% (w/v) agarose in TAE buffer, ethidium bromide for visualization, 1 kb DNA standard ladder, agarose electrophoresis chamber systems, and appropriate power supply.
3. Template genomic DNA (gDNA) from a wild-type strain such as ATCC 33270, plasmid templates containing appropriate selectable markers, such as the kanamycin resistance cassette (*aphAIII*) (e.g., pWVTK) and erythromycin resistance cassette (*ermB*) (e.g., pJY4164) [9–11]. Antibiotic cassette primers (5′-3′): aph-F- CCCAGGACAATAACCTTATAGC, aph-R- TCGATACAAATTCCTCGTAGGC, erm-F- GACGGTTCGTGTTCGTGCTGAC, erm-R- GCGACTCATAGAATTATTTCCTCC.
4. Gibson assembly master mix (e.g., New England Biolabs).
5. 0.1 mm glass or zirconia/silica beads (e.g., BioSpec).
6. 1.5 mL polypropylene tubes (e.g., Eppendorf).
7. Vortex mixer.
8. PCR cleanup kit (e.g., QIAquick, Qiagen).
9. Microcentrifuge (e.g., Eppendorf model 5418 or equivalent).

2.3 DNA Transformation

1. Purified DNA from PCR amplification of Gibson assembly reaction.
2. Anaerobic chamber (85% N_2, 10% CO_2, 5% H_2) at 37 °C.
3. sBHI agar (+/− appropriate antibiotic).
4. 1.5 mL polypropylene tubes.
5. Vortex mixer.
6. Spectrophotometer with compatible disposable 1.5 mL cuvettes.

2.4 Mutant Screening and Selection

1. sBHI agar (+/− appropriate antibiotic).
2. PCR and agarose gel electrophoresis supplies.
3. Flanking forward and reverse primers (to be designed specifically for the target gene of interest).
4. Polyester tipped swabs.
5. 0.1 mm glass or zirconia/silica beads.
6. Disposable inoculating loops (1 μL).
7. 1.5 mL polypropylene tubes.
8. sBHI media with 25% (v/v) glycerol (sterile).
9. Cryovials.
10. Vortex mixer.

3 Methods

3.1 Bacterial Culture

The following method describes the culturing of *P. micra* for the purpose of generating mutants to study cell wall biogenesis and function (*see* **Note 3**).

1. From −80 °C freezer glycerol stocks of *P. micra* (wild-type reference strain ATCC 33270 [8] or other naturally competent wild-type strain [7]), scrape a portion from the frozen surface using a sterile pipette tip and spot onto the sBHI agar surface. Using a sterile loop, quadrant streak the agar plate to isolate individual bacteria (Fig. 1ai).
2. Incubate in an anaerobic chamber (85% N_2, 10% CO_2, 5% H_2) at 37 °C for 4–7 days (*see* **Note 4**).

3.2 Mutagenesis Construct Assembly

Most prokaryotic mutagenesis constructs are assembled by subcloning foreign DNA sequences into plasmid vectors hosted by cloning strains of *E. coli*. However, this process can be quite time-consuming and may be especially problematic when attempting to clone A + T-rich DNA, due to the frequent instability of such constructs in *E. coli* [12]. *P. micra* has a G + C content of only ~28% [7], which is significantly lower than that of *E. coli* (~50%)

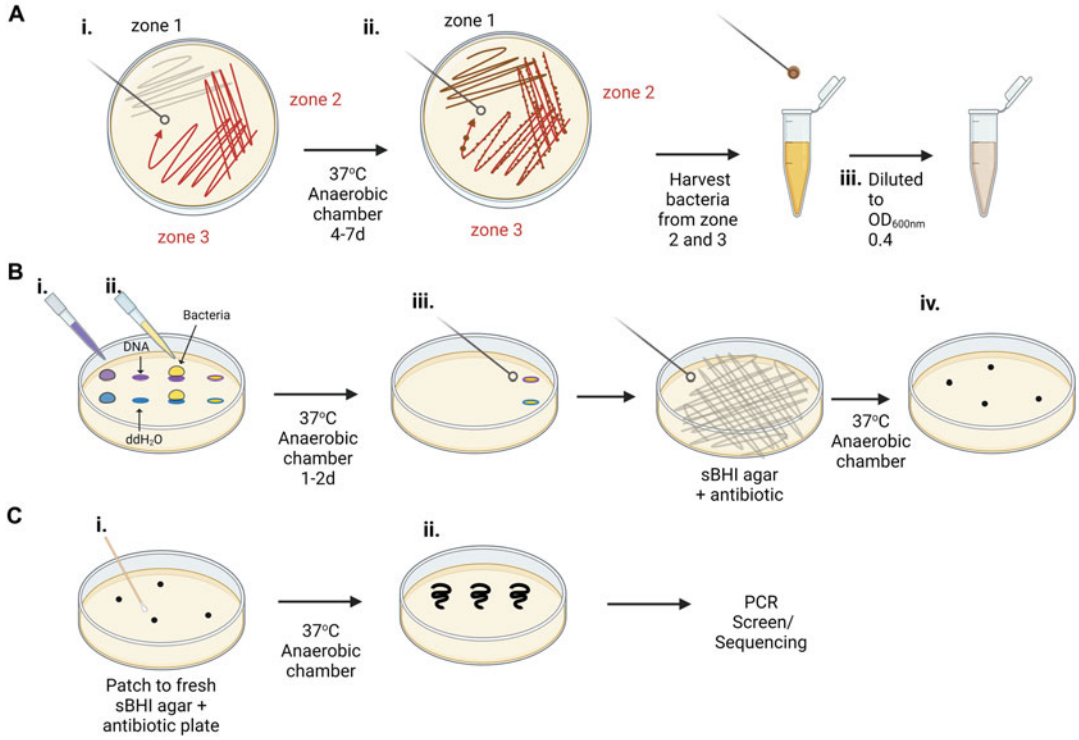

Fig. 1 Overview of *P. micra* growth, transformation, and selection. (**a.i**) Serial streaking to obtain growth of (**a.ii**) single colonies. For transformation, bacterial colonies in zones 2 and 3 (in red) are harvested and (**a.iii**) diluted to an $OD600_{nm}$ of 0.4 in sBHI. DNA (purple) and negative controls (blue) are (**b.i**) spotted on sBHI agar and allowed to absorb. Diluted bacterial suspensions are (**b.ii**) spotted (yellow) directly on top of experimental DNA and control spots and allowed to absorb. Cultures are incubated in anaerobic conditions at 37 °C for 1–2 days. Bacteria are (**b.iii**) harvested and spread onto sBHI agar plates supplemented with the appropriate antibiotic. Plates are incubated in anaerobic conditions at 37 °C for 4–8 days. (**b.iv**) Single transformants can be (**c.i**) patched to a fresh sBHI antibiotic plate (**c.ii**) for growth and screening

[13]. Therefore, *P. micra* DNA is often difficult to clone in *E. coli*, especially when using typical high copy-number cloning vectors. Additionally, cloned mutagenesis constructs may also encode gene products that are toxic to *E. coli*, which can add an additional layer of complication. To circumvent these issues, the following cloning-independent methodology employs only PCR and Gibson-based assembly for the creation of mutagenesis constructs [14], thus eliminating the need for intermediate hosts like *E. coli*.

1. To generate template gDNA for subsequent PCR reactions, harvest a loopful of bacteria from zones 2 and 3 of a quadrant-streaked plate of *P. micra* (*see* Subheading 3.1, **step 2**) using a sterile loop (Fig. 1aii) (*see* **Note 5**). Transfer to 0.5–1 mL of ddH_2O in a polypropylene tube and add ~25% volume of 0.1 mm glass or zirconia/silica beads. Vortex at high setting for ≥2 min to liberate DNA. Pellet beads and large cellular

debris by centrifugation at ≥16,000 × *g* for 2 min, collect supernatant, and transfer to a clean tube.

2. PCR primers need to be designed to amplify each desired fragment (*see* **Note 6**). Each fragment should include ~15–25 bp of complementary homology (*see* **Note 6**) to the adjacent fragment (Fig. 2ai, bi) and yield a melting temperature (T_m) of >53 °C.
3. For mutagenesis constructs, the PCR amplicons containing the regions of homology to chromosomal DNA for double-crossover should be between 1.0 and 2.5 kb (*see* **Note 7**).
4. For the generation of complementation constructs, skip to **step 5**. For the generation of deletion constructs, set up the PCR reactions for each fragment using the selected polymerase, corresponding buffer(s), dNTPs, and specific primers. For PCR amplicons representing homologous flanking regions, use the appropriate primer pairs (1F/1R and 2F/2R, primer pairs to be designed specifically for the target gene of interest) and template gDNA for amplification (Fig. 2ai). For the PCR amplicon representing the *ermB* cassette, use the primers erm-F/R (erm-F- GACGGTTCGTGTTCGTGCTGAC , erm-R- GCGACTCATAGAATTATTTCCTCC) with *ermB* template for amplification (Fig. 2ai). The gDNA template should constitute ≤1/20 of the total reaction volume (proceed to **step 6**).
5. For the generation of complementation constructs, set up the PCR reaction for each fragment using polymerase, corresponding buffer(s), dNTPs, and specific primers. For PCR amplicons representing flanking regions to the *tuf* insertion site (*see* **Note 6**), use the primer pairs (3F-ACAAAGGG GTTCAACCTCTATTG /3R, primer to be designed specifically for the target gene of interest and 5F- GCCTAC GAGGAATTTGTATCGATTATACTGATTATTTTAAAAGG GAATCATAGTG /5R-TGATGTAGGTCTTTCAGAAAAAGG) and the appropriate primer pair (4F (*see* **Note 8**)/4R) designed specifically for the target gene of interest, using template gDNA for amplification (Fig. 2bi). For the PCR amplicon representing the *aphIII* cassette, use primers aph-F/R (aph-F- CCCAGGACAA TAACCTTATAGC , aph-R- TCGATACAAATTCCTCG TAGGC) with *aphIII* template for amplification (Fig. 2bi). The DNA template should constitute ≤1/20 of the total reaction volume.
6. Analyze by agarose gel electrophoresis to confirm the expected molecular weights of all PCR amplicons.
7. Purify PCR amplicons using a PCR cleanup kit and then determine their concentrations with a spectrophotometer.

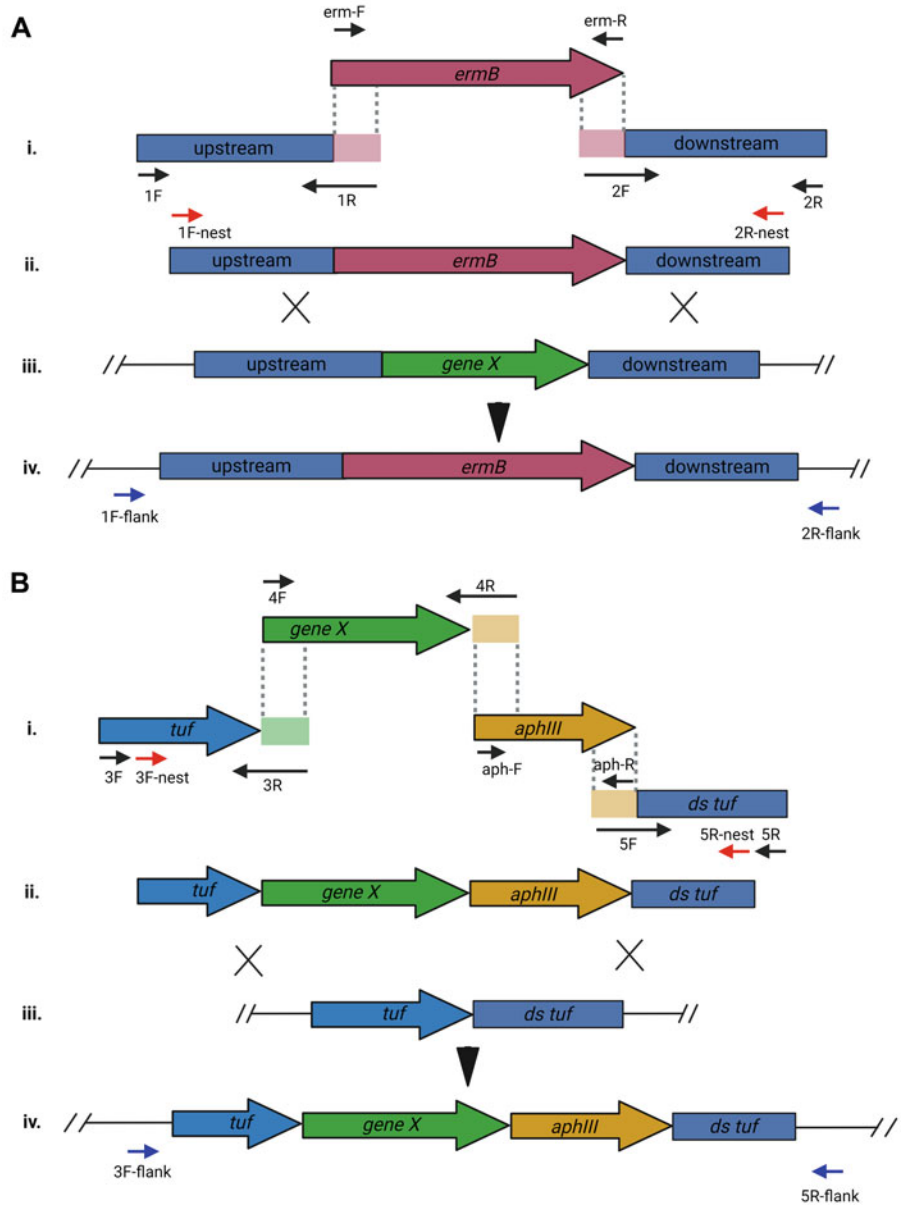

Fig. 2 DNA mutagenesis construction using PCR and Gibson assembly. Complementary overlapping sequences to adjacent fragments are created by adding a 5′ tail to specified primers (dashed lines). For gene deletion by allelic replacement (**a.i**), PCR amplicons of upstream and downstream fragments homologous (blue) to the target gene (green) and *ermB* cassette (red) are assembled and amplified using nested primers (red arrows) and then (**a.ii**) inserted into the *P. micra* chromosome through (**a.iii**) homologous recombination (**a.iv**), yielding erythromycin-resistant transformants. For complementation constructs (**b.i**), PCR amplicons of upstream and downstream fragments homologous (blue) to the *tuf* locus insertion site, the *aphIII* cassette (yellow), and target gene (green) are assembled and amplified using nested primers (red arrows) and then (**b.ii**) inserted into the *P. micra* chromosome through (**b.iii**) homologous recombination (**b.iv**), yielding kanamycin-resistant transformants. Primers used for PCR verification of genotypes are illustrated by blue arrows

8. Assemble the fragments as per the manufacturer's instructions for the selected Gibson assembly kit.
9. For PCR amplification of Gibson-assembled constructs, set up the reactions using polymerase, corresponding buffer(s), dNTPs, and nested primers 1F-nest/2R-nest (primers designed specifically for the target gene of interest) (Fig. 2ai) or 3F-nest- TTCTCACGTTGAATATGAAACTGC /5R-nest-AAGTCCTCCATAAACAATACTTAAAC (Fig. 2bi) using the respective Gibson assembled DNA from step 8 as a template (should constitute about 1/100th the volume of total reaction).
10. Analyze by agarose gel electrophoresis to confirm the expected molecular weight of the resulting PCR amplicon.
11. Purify the assembled PCR product using the PCR cleanup kit and determine its concentration using a spectrophotometer.

3.3 DNA Transformation

This procedure requires the natural competence ability of *P. micra* [7]. Below, we describe a method that employs *P. micra* natural transformation to insert constructs onto the chromosome by an allelic replacement double-crossover event (Fig. 2aii, iii, iv and b.ii, iii, iv).

1. Grow *P. micra* on sBHI agar as described (*see* Subheading 3.1, **step 2**).
2. While working within an anaerobic chamber, use a disposable inoculating loop to collect the bacteria from the sBHI plate and transfer to 1 mL of prereduced sBHI broth in a 1.5 mL polypropylene tube (Fig. 1aii) (*see* **Note5**). Swirl the loop to facilitate the release of bacteria into the broth. Vortex the suspension at high setting for 2 min. Dilute 1/10 by adding 0.1 mL of bacterial suspension into 0.9 mL of sBHI broth in a polypropylene tube and then vortex for 30 s. Transfer the mixture to a cuvette. Blank the spectrophotometer at λ600 nm optical density (OD_{600}) using sBHI broth and determine the OD_{600} for both, the diluted bacterial suspension, and the original suspension (×10).
3. Dissolve 1–2 μg of PCR-amplified DNA in ddH_2O to a final volume of 20 μL and spot onto a fresh sBHI agar plate. Include a spot containing 20 μL ddH_2O as a negative control and allow it to fully absorb into the agar (Fig. 1bi).
4. While the DNA/water spots are absorbing, calculate the dilution of the original bacterial suspension necessary to achieve a final OD_{600} of 0.4 (Fig. 1aiii). Dilute the original bacterial suspension with prereduced sBHI accordingly and vortex for 1 min.

5. Overlay 20 μL of the *P. micra* suspension (OD_{600} of 0.4) onto each fully absorbed DNA and ddH_2O spot and then allow it to fully absorb into the agar as well (Fig. 1bii).
6. Incubate in anaerobic conditions at 37 °C for 1–2 days.
7. Following the incubation period, harvest cells using an inoculating loop and spread onto sBHI agar plates containing the appropriate antibiotic (Fig. 1biii).
8. Incubate in anaerobic conditions at 37 °C. Monitor daily for colony growth, typically 4–8 days (Fig. 1biv) (*see* **Notes 9** and **10**).
9. Subculture transformants by harvesting single colonies using a polyester tipped swab and transferring to a fresh sBHI agar plate containing the appropriate antibiotics (Fig. 1ci,ii).
10. Incubate in anaerobic conditions at 37 °C for 4–7 days.

3.4 Mutant Screening

After restreaking single colonies of *P. micra* transformants, PCR analysis and sequencing should be performed to confirm the desired genotypes.

1. Harvest a portion of each subcultured transformant (Fig. 1cii) from the agar plate using inoculating loops and suspend in 0.2 mL of ddH_2O.
2. To lyse bacteria, add ~25% volume of 0.1 mm glass or zirconia/silica beads to the suspension and vortex at high setting for 2 min.
3. Pellet beads and large cellular debris by centrifugation at ≥16,000 × *g* for 2–4 min, collect supernatant, and transfer to a clean polypropylene tube. This will serve as the template for PCR analysis.
4. Prepare PCR reactions with polymerase, specific buffers, dNTPs, and flanking primers 1F-flank/2R-flank (Fig. 2aiv) for the screening of gene deletion mutants and 3F-flank/5R-flank (Fig. 2biv) for the screening of complementation insertion mutants using their respective templates, which should constitute ≤1/20 of the total reaction volume.
5. For detecting allelic replacement mutants and/or gene insertions, one can simply perform the series of targeted PCR reactions in step 4, followed by agarose gel electrophoresis. Primer combinations of 1F-flank/erm-R and erm-F/2R-flank are additionally recommended for allelic replacement mutants (Fig. 2). Further sequencing of the resulting PCR products can be performed for final confirmation.
6. To make freezer stocks of verified mutant bacteria, resuspend the remaining subcultured bacteria (*see* Subheading 3.3, **step 10**) in a cryovial containing sBHI with 25% (v/v) glycerol. Vortex for 10 s and freeze at ≤−80 °C.

4 Notes

1. All growth media should be prereduced in an anaerobic chamber ≥24 h before use.
2. Clinical isolates of *P. micra* display varying levels of natural competence. Strains of interest can be tested for natural competence beforehand [7].
3. *P. micra* is typically classified as a Biosafety level 1 (BSL-1) organism and should be handled under BSL-1 guidelines and the guidelines of your institution using proper personal protective equipment. Sterile technique and anaerobic conditions should be maintained while handling bacteria at each step.
4. If the colony density derived from the freezer stock is low, use an inoculating loop to pick 5–10 single colonies from the primary growth plate and streak onto a fresh sBHI agar plate (no antibiotics) for single colonies and allow to grow 4–7 days.
5. Collect bacteria from zones #2 and #3 of the streak, representing robustly growing colonies.
6. Sequence variations between strains of *P. micra* should be taken into consideration in primer design. Primers listed here are specific to ATCC strain 33270. Primer complementarity to adjacent homologous regions should have a $T_m \geq 53$ °C ($T_m = 2(A + T) + 4(G + C)\text{-}5$).
7. Increasing homologous flanking regions (from 1 kb to 2.5 kb) can significantly increase transformation efficiency.
8. It is recommended to include the ribosomal binding site (RBS) and a 4–9 nt spacer before the start codon of your gene of interest, which can be directly incorporated into the forward primer. An example RBS (italics), spacer, and start codon (underlined): 5′-*AGGAGG*AAGCAATG-3′.
9. Growth of bacteria from the negative control spot may indicate the antibiotic concentration needs to be empirically adjusted.
10. For extended incubation times, humidification may be necessary. Cultures can be placed in a perforated container such as a clean pipette tip box containing a moistened paper towel.

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Chapter 6

A New Method for Gene Deletion to Investigate Cell Wall Biogenesis in *Fusobacterium nucleatum*

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Abstract

Controlled septal peptidoglycan hydrolysis is vital for bacterial cell division, preserving cellular integrity and facilitating proper daughter cell separation. In *Escherichia coli*, the FtsEX ABC system governs cell wall hydrolase activation by regulating the EnvC activator. However, the processes underlying cell division in the Gram-negative oral bacterium *Fusobacterium nucleatum* are poorly understood, mainly due to its well-known genetic intractability. Herein, we provide a step-by-step procedure for a new gene deletion method in *F. nucleatum*, focusing on the *ftsX* gene as a target. This novel approach exploits the HicAB toxin-antitoxin system, using HicA as a counter-selective marker to enable efficient and precise gene deletion. By implementing this technique, we successfully demonstrated its applicability in *F. nucleatum*, providing new insights into this important microorganism's cell division process. Furthermore, this advanced gene deletion technique offers a valuable resource for future investigation into the functional characterization of genes involved in cell wall biogenesis in *F. nucleatum* and other genetically intractable microorganisms.

Key words *Fusobacterium nucleatum*, In-frame deletion, Cell division, Cell wall biogenesis, Gram-negative anaerobe

1 Introduction

Fusobacterium nucleatum, an obligately anaerobic, Gram-negative bacterium, is a common inhabitant of the human oral cavity and gastrointestinal tract. It is a key component of the subgingival dental biofilm, comprising over 400 bacterial species [1, 2]. *F. nucleatum* significantly impacts the onset and progression of periodontal disease through its physical interactions with the host and other bacterial species within the oral biofilm [2–4]. Moreover, *F. nucleatum* has been implicated in various extra-oral diseases, including preterm birth [5, 6] and colorectal cancer [7–10]. Research has demonstrated that *F. nucleatum* plays a crucial role in developing these diseases, highlighting that it is an important target for developing treatments and therapies.

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Gram-negative bacteria, such as *F. nucleatum*, possess an outer membrane (OM) containing lipopolysaccharide (LPS) and a layer of peptidoglycan (PG) constituting the bacterial cell wall [11]. The cell wall is essential for maintaining bacterial shape, providing environmental protection, and contributing to its pathogenicity [12–14]. As a dynamic structure, the cell wall undergoes simultaneous synthesis and degradation during cell growth. Striking a balance between these two opposing processes is vital for preserving a robust and intact bacterial cell wall. Gaining insight into the molecular mechanisms underpinning the assembly of the Gram-negative cell wall is crucial for developing new treatments that effectively combat drug-resistant infections. While the individual pathways for PG and OM synthesis and assembly are well-characterized in other bacteria, little is known about the cell wall biogenesis in *F. nucleatum*. The FtsEX complex is an ABC transporter in *E. coli* that controls the activity of periplasmic peptidoglycan amidases by interacting with the murein hydrolase activator, EnvC [15]. We previously examined the roles of FtsX and EnvC in cell division and biofilm formation in *F. nucleatum*. Deleting either gene resulted in changes to the surface morphology and the cell envelope structures, indicating their involvement in cell wall formation and cell division [16].

However, the method for creating these deletions relies on *galK* as a counterselection marker. While the *galK*-based in-frame deletion method is effective, it has an inherent limitation: it necessitates a host strain lacking the *galk* gene. Since *galK* plays a vital role in cell envelope maintenance in bacteria [17, 18], this method is not ideal for studying genes involved in cell wall biogenesis in *F. nucleatum*. To address this issue, we recently developed a HicA-based counterselection marker for *F. nucleatum* and successfully deleted genes in the wild-type background of *F. nucleatum* [19]. In this chapter, we provided a detailed protocol for using the HicA toxin-based markerless in-frame deletion method, illustrated by the deletion of the *ftsX* gene in *F. nucleatum*.

2 Materials

2.1 Preparation of Agar or Liquid Medium for the Growth of F. nucleatum

1. Wild-type reference strain ATCC 23726.
2. Supplemented Tryptic Soy Peptone (TSP) medium: Weight 30 g of Tryptic Soy Broth powder (BD or equivalent), dissolve it in 1 liter of distilled water. Add 10 g of Bacto-peptone (BD or equivalent) to the solution and stir until it is completely dissolved. Autoclave the media at 121 °C for 20 min. Allow it to cool to room temperature before use.
3. Working TSPC broth: A pipette transfers 8 mL of TSP medium into 16 × 150 mm culture tubes (VWR). Next, withdraw

0.4 mL of 1% cysteine stock solution (or diluted ten-fold from the 10× cysteine stock) using a 1 mL syringe and add it to the TSP in the tubes. Do not shake the tubes.

4. Freezer stocks (−80 °C) of *F. nucleatum* in 3% tryptic soy broth (TSB) supplemented with 1% Bacto™ peptone plus 0.05% freshly made cysteine (TSPC) with 25% (v/v) glycerol in cryovials.
5. Freezer stocks (−80 °C) of *E. coli* DH5α pBCG02 vector [19] in Luria-Bertani (LB) broth with 15 μg/mL chloramphenicol with 25% (v/v) glycerol in cryovials.
6. TSPC agar plates. Dissolve 30 g of Tryptic Soy Broth (BD or equivalent) into 1 liter of distilled water with stirring. Next, add 10 g of Bacto peptone and 15 g of agar while continuing to stir. Autoclave media for 20 min at 121 °C, then allow to cool down to 50–55 °C in a water bath. Add 10 mL of 10× cysteine solution and mix thoroughly. Dispense the mixture into Petri dishes and allow it to solidify.
7. Syringe filters (0.22 μm) and compatible 10 mL syringe.
8. L-cysteine hydrochloride monohydrate.
9. 10× cysteine solution (100 g/L): Dissolve 10 g of L-cysteine into 100 mL of double-distilled water (ddH_2O). Place the resulting solution in an anaerobic chamber to eliminate oxygen. Subsequently, dispense the L-cysteine solution into Hungate anaerobic tubes in 10 mL aliquots. After closing the lids of the tubes, remove them from the anaerobic chamber and sterilize them by autoclaving at 121 °C for 15 min. Once sterilized, the cysteine stocks can be stored in a room template on the bench for further use (*see* **Note 1**).
10. Hungate anaerobic tube 16 × 125 mm (Chem Glass Life Science).
11. Butyl rubber stopper and screw cap having a 9 mm opening.
12. 1 mL disposable syringe with needle.
13. For the TSPA agar selection plates: Add the appropriate amount of antibiotic stock solutions in the media and mix gently to achieve the final concentration of 5 μg/mL (w/v) thiamphenicol or 2 mM of theophylline inducer. And then, dispense the mixture into Petri dishes. The plates are allowed to solidify at room temperature before selection.
14. Tryptic Soy Broth (TSB).
15. Bacto™ peptone.
16. Bacto agar.
17. Luria-Bertani (LB) Broth, Powder (Miller's): Dissolve 25 g of LB broth powder in 1 liter of distilled water, stirring gently until completely solubilized. Next, dispense the media into

appropriate containers and loosen the caps. Autoclave the containers for 15 minutes at 121 °C.

18. LB agar plates with 20 μg/ml chloramphenicol.
19. Chloramphenicol stock: 30 mg/ml (*see* **Note 2**).
20. Thiamphenicol stock: 5 mg/ml (*see* **Note 2**).
21. 40 mM of theophylline stock: Weigh 0.228 g theophylline and dissolve it into 40 mL of TSP media. After filter sterilization, store the solution at 4 °C (*see* **Note 2**).
22. 1.5 mL centrifuge tube.
23. Sterile Petri dishes (100 mm × 15 mm).
24. 16 mm glass culture tubes.
25. 16 mm culture tube caps.
26. Standard laboratory pipette tips.
27. Standard laboratory pipette set.
28. 1.5 mL cryogenic tubes.
29. Disposable inoculating loops.
30. Anaerobic chamber (80% N_2, 10% CO_2, 10% H_2) at 37 °C.

2.2 Construction of pBCG02a & pBCG02a-ΔftsX

1. Freezer stocks (−80 °C) of *E. coli* DH5*α* pBCG02.
2. Purified genomic DNA from *F. nucleatum* subsp. *nucleatum* ATCC 23726.
3. PCR supplies: molecular biology grade nuclease-free water, 2× Hot Start PCR master mix from TaKaRa (catalog no. R405A), APEX 2× Taq RED Master Mix or equivalent PCR reagents, thermocycler, and compatible PCR tubes.
4. Agarose gel electrophoresis supplies: TAE buffer (40 mM tris base, 20 mM acetic acid, 1 mM EDTA), 1.5% (w/v) agarose in TAE buffer, ethidium bromide for visualization, 1 kb DNA standard ladder, agarose electrophoresis chamber systems, and appropriate power supply.
5. Gibson assembly master mix kit (e.g., New England Biolabs).
6. Vortex mixer.
7. PCR cleanup kit (e.g., QIAquick, Qiagen).
8. DNA gel cleanup kit (e.g., QIAquick, Qiagen).
9. Microcentrifuge (e.g., Eppendorf).
10. Ice.
11. Custom primers (see primer design details below):
    (i) P1: TTTTGGATCCTTTTGGTACCGTAGCTTCCATA CATATCACACTCCAG.
    (ii) P2: AAAAGGATCCAAAAGTCGACGTCCTAGAACTA GTGAAATTTCCG.

(iii) P3: GAGTGTGATATGTATGGAAGCTACGGTACAG AAATCTTAAAGGAAGAAAAAAAGAATGT.

(iv) P4: ATTTCCTTTTTTCAGCTTCTATTTTCATGAAA ATATTTAATGCTACAACTGTAATT.

(v) P5: GTAGCATTAAATATTTTCATGAAAATAGAAGCT GAAAAAAGGAAATTAGACAG.

(vi) P6: GCCGGAAATTTCACTAGTTCTAGGACGATAAC TGTTCCTGCCTTAGAAGCAACTACTGG.

12. DNA Digest regents:
    (i) Restriction enzymes of choice (KpnI and SalI).
    (ii) 10× Cutsmart Buffer (NEB).
13. Gel extraction kit (e.g., QIAquick, Qiagen).
14. Razor blade.
15. Plasmid DNA minipreps purification kit (e.g., QIAquick, Qiagen).
16. Water bath set to 42 °C.
17. Heating block set to 60 °C.
18. Tabletop centrifuge.
19. Anaerobic incubator maintained at 37 °C.
20. NanoPhotometer N60 (IMPLEN) instrument for DNA concentration measurement.
21. *E. coli* DH5α chemically competent cells (NEB).
22. Anaerobic chamber (80% N_2, 10% CO_2, 10% H_2) at 37 °C.

2.3 Transformation of pBCG02a-ΔftsX in F. nucleatum and Mutant Screening

1. *F. nucleatum* competent cells [20].
2. Purified plasmid pBCG02a-Δ*ftsX*.
3. Pre-chill 0.1 mm Biorad gene pulser cuvettes at −20 °C.
4. Gene Pulser Xcell modular electroporation systems.
5. TSPC agar plates with 5 μg/ml thiamphenicol.
6. TSPC broth with 5 μg/ml thiamphenicol.
7. TSPC broth without antibiotics.
8. TSPC agar plates with 2 mM theophylline.
9. TSPC agar plates without antibiotics.
10. 1.5 mL polypropylene tubes.
11. Vortex mixer.
12. Forward and reverse primers.
13. Polyester-tipped swabs.
14. Disposable inoculating loops.

15. PCR supplies: molecular biology grade double distilled water (ddH_2O), 2× Hot Start PCR master mix from TaKaRa, APEX 2× Taq RED Master Mix or equivalent PCR reagents, thermocycler, and compatible PCR tubes.
16. Agarose gel electrophoresis supplies: TAE buffer (40 mM tris base, 20 mM acetic acid, 1 mM EDTA), 1.5% (w/v) agarose in TAE buffer, ethidium bromide for visualization, 1 kb DNA standard ladder, agarose electrophoresis chamber systems, and appropriate power supply.
17. 15 mL propylene tube.
18. 2.0 mL cryogenic tubes.
19. Anaerobic chamber (80% N_2, 10% CO_2, 10% H_2) at 37 °C.

3 Methods

F. nucleatum strains are grown in 3% tryptic soy broth (TSB) supplemented with 1% Bacto™ peptone plus 0.05% freshly made cysteine (TSPC) or on TSPC agar plates in an anaerobic chamber filled with 80% N_2, 10% H_2, and 10% CO_2. TSPC agar plates are always freshly made under sterile conditions before use; plates are air-dried inside a biosafety cabinet that has filtered, continuous airflow (*see* **Note 3**).

When starting fusobacterial growth from glycerol stocks, it is possible to inoculate media or spread onto plates under aerobic conditions. However, cultured plates must be rapidly transferred to an anaerobic chamber. To prevent dehydration, wrap the plates with parafilm once they are in an anaerobic environment. The duration needed for the optimal growth of colonies on TSPC agar depends on the strains, but typically, it takes 2–3 days.

3.1 Construction of pBCG02a

pBCG02 (7289 bp) is an *E. coli*/*F. nucleatum* shuttle vector expressing a truncated *hicA* gene (3A) from *F. periodonticum* 33,693 under the control of the $rpsJ_{FP}$ promoter and its ribosome binding site was replaced by riboswitch element E from pRECas2 [21]. To facilitate the gene deletion method, we created a smaller, suicidal vector called pBCG02a (4861 bp) (Fig. 1a). This vector does not contain *repA* and *oriFn*, but it does have KpnI, BamHI, and SalI restriction sites that simplify the downstream gene cloning process. The construction of pBCG02a was carried out according to the following procedure:

1. Use a Plasmid DNA minipreps purification kit to isolate the pBCG02 plasmid from the *E. coli* strain.
2. Use 2× Hot Start PCR master mix (or equivalent PCR reagent) from TaKaRa (catalog no. R405A) for high-fidelity PCR

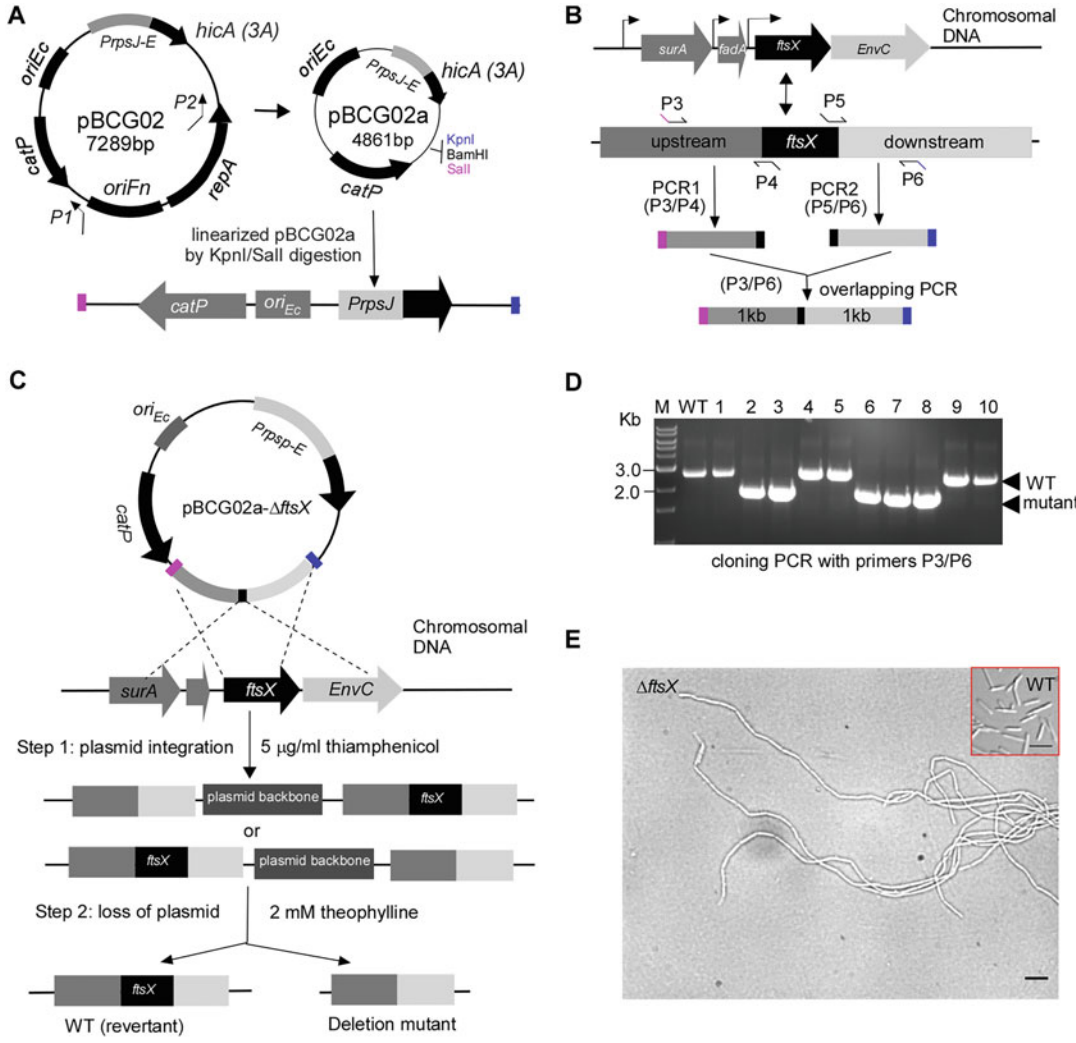

Fig. 1 Generation of an *ftsX* gene deletion *F. nucleatum* wild-type background. This procedure applies to any gene of interest, including *ftsX*. (**a**) An example of *ftsX* open reading frame (ORF) with upstream- and downstream genes. Two primers (P3/P4 and P5/P6) are used in PCR reactions generating a 1 Kb upstream fragment and downstream fragment, respectively. The upstream fragment and downstream fragment are adjoined by overlapping PCR. (**b**)**.** Graphic representation of the construction of derivative of pBCG02 as smaller suicidal vector pBCG02a (4861 bp) lacking *repA* and *oriFn*, and containing KpnI, BamHI, and SalI restriction sites. Linearized non-replicative vector backbone pBCG02a, used to generate unmarked in-frame gene deletions in *F. nucleatum,* is generated by KpnI and SalI digestion. (**c**)**.** Approximately 1 kb fragments homologous to the upstream and downstream regions of the *ftsX* were amplified by PCR. They have ligated adjacently on the vector backbone pBCG02a via the Gibson assembly procedure (see details in context). The plasmid pBCG02-Δ*ftsX* generated an unmarked *ftsX* deletion mutant in the wild-type background by a two-step allelic exchange. (**d**) The screen of *ftsX* deletion mutants by colony PCR with the primer pairs P3/P6. Lane 1–10, 10 μL of PCR products of nine colonies randomly selected from the TSPC plate. Five of the ten chosen random colonies were deletion mutants, while five reverted to wild type during the second recombination event. Bands of approximately 2 kb indicate *ftsX* deletion, whereas bands of about 2.7 kb indicate a wild-type (WT) genotype. (**e**) *F. nucleatum* Δ*ftsX* strain and WT was grown in a TSPC medium for 16 hours of growth in the anaerobic chamber. —Phase-contrast microscopy recorded cell shape. Deletion of *ftsX* resulted in filamented cell morphology compared to wild type (upper right corner inserts): bars, 2 μm

amplification of the pBCG02 backbone. Set up the following PCR reaction on ice. Generally, prepare two tubes for each reaction.

PrimeStar Max Premix (2×) 25 μL
P1 2 μL
P2 2 μL
Template (pBCG02) 50 ng
Nuclease-free water to 50 μL

3. Analyze by agarose gel electrophoresis to confirm the expected molecular weight of PCR amplicon.
4. Purify PCR products using a PCR cleanup kit and determine their concentrations using a nanodrop instrument.
5. Adjoin the two ends of the pBCG02 amplicon as per the manufacturer's instructions for the Gibson assembly kit to produce pBCG02a (4861 bp).

   Set up the following reaction on ice:

pBCG02 vector backbone	100 ng (X)μL
Gibson assembly master mix (2×)	10 μL
Deionized H_2O	10-X μL
Total volume	20 μL

6. Sample was incubated in a thermocycler at 50 °C for 15 minutes. Following incubation, samples were stored on ice or at −20 °C for subsequent transformation.
7. Transform NEB 5α Competent *E. coli* cells (provided with the kit) with 5 μL of the assembly reaction, following the transformation protocol.
8. Transform into chemically competent cells of *E. coli* DH5α.
   (i) Thaw *E. coli* chemically competent DH5α strain on ice (~10 min).
   (ii) Add 5 μL assembly to 50 μL *E. coli* chemically competent DH5α strain. Mix gently by pipetting up and down or flicking the tube 4–5 times. Do not vortex.
   (iii) Place cells back onto the ice for 15–40 min.
   (iv) Heat shock cells for 50 sec at 42 °C.
   (v) Transfer tubes back onto the ice for 2 min.
   (vi) Add 950 μL SOC medium to cells and transfer to a 16 mm glass culture tube.
   (vii) Incubate with shaking for 1 h at 37 °C.

(viii) Warm selection plates to 37 °C. If needed, plate 100 μL and 200 μL onto LB plates with 20 μg mL^{-1} chloramphenicol.

(ix) Incubate both plates overnight at 37 °C.

(x) Grow a few colonies in LB medium with 20 μg mL^{-1} chloramphenicol.

(xi) Use a Plasmid DNA minipreps purification kit to purify plasmid DNA and verify by sequencing.

(xii) Determine plasmid DNA concentrations using a Nanodrop instrument (or equivalent).

3.2 Construction of pBCG02a-ΔftsX

1. Design and order primer pairs to amplify 1 kb regions upstream and downstream of *ftsX* gene (*see* **Note 4**). Four primers are required for the cloning procedure described below (*see* Subheading 2). Primers P3 and P4 amplify the upstream region of the *FtsX* gene, while primers P5 and P6 amplify the downstream region *ftsX* (*see* **Note 4**). Each fragment should include ~15–25 bp sequence of complementary homology to the adjacent fragment (*see* Fig. 1a, b). A 2× PrimeSTAR Max Premix from TaKaRa (catalog no. R405A) was used for high-fidelity PCR amplification of the *ftsX* upstream and downstream regions (*see* Fig. 1b).
2. Set up the following PCR reaction on ice as described in Subheading 3.1, Construction of pBCG02a, **step 1**. Use purified genomic DNA from ATCC 23726 or washed *F. nucleatum* cells as a DNA template to PCR amplify the upstream and downstream regions of the *FtsX* gene.
3. Analyze by agarose gel electrophoresis to confirm the PCR amplicons.
4. Purify PCR amplicons using a PCR cleanup kit and determine their concentrations using a Nanodrop. Adjust the concentration of the PCR products to 50 ng/μL for subsequent experimental steps.
5. Next, perform an overlapping PCR to join the upstream and downstream PCR fragments. Prepare a PCR reaction containing 25 ul of PrimeSTAR Max Premix (2×), 2 μL for each upstream and downstream PCR product, and 18 μL H_2O. Follow the recommended PCR program outlined below:

   98 °C for 8 min.

   10 cycles of 98 °C for 10 sec, 53 °C for 20 sec, and 72 °C for 2 min.

   Upon completing this program, add 2 μL of primer P3 and 2 μL of primer P6. Proceed with the following program:

   30 cycles of 98 °C for 10 sec, 53 °C for 20 sec, and 72 °C for 2 min.

   72 for 10 min

6. Perform agarose gel electrophoresis to confirm the PCR amplicon.
7. Purify PCR amplicons using a PCR cleanup kit and then determine their concentrations with a nanodrop and adjust the concentration of the overlapping PCR product to 100 ng/μL for subsequent experimental steps.
8. Digest the pBCG02a vector (1 μg) with KpnI and SalI (*see* Fig. 1a).

   Digestion reaction (final volume of 100 μL) at 37 °C for 2 h:

DNA	Up to 1 μg
10× CutSmart buffer	10 μL
Restriction enzyme 1 (KpnI)	4 μL
Restriction enzyme 2 (SalI)	4 μL
Nuclease-free water	Up to 100 μL

9. Purify digested products using a DNA Gel Extraction Kit and determine their concentrations using a nanodrop.
10. Perform agarose gel electrophoresis to confirm the expected molecular weight of digested vector.
11. Assemble the fragments per the manufacturer's instructions provided with the Gibson assembly kit. Set up the following reaction on ice:

pBCG02 vector backbone	100 ng (X)μL
ftsXup and *ftsXdn* DNA fragment	300 ng (Y)μL
Gibson assembly master mix (2×)	10 μL
Deionized H_2O	10-X-Yμl
Total volume	20 μL

12. Incubate samples in a thermocycler at 50 °C for 20 min. Following incubation, store samples on ice or at −20 °C for subsequent transformation.
13. Transform *E. coli* 5α competent (provided with the kit) with 5 μL of the assembly reaction, following the transformation protocol in Subheading 3.1, Construction of pBCG02a, **step 8**.
14. Screen for mutant clones via colony PCR with primers P3/P6.

Colony PCR reaction (final volume of 25 μL).

APEX 2× Taq RED Master Mix	12.5 μL
Primer P3	1.25 μL
Primer P6	1.25 μL
Nuclease-free water	Up to 25 μL

Verify the amplicons by gel electrophoresis using 5 μL PCR products.

15. Use a Plasmid DNA minipreps purification kit to purify plasmid DNA and determine plasmid DNA concentrations using a Nanodrop instrument (or equivalent). Send plasmid for sequencing.

3.3 Transformation of pBCG02a-ΔftsX in F. nucleatum and Mutant Screening

1. Transform plasmid into *F. nucleatum* strain ATCC23726 (to obtain plasmid integration strain, *see* Fig. 1c, **step 1**).
   Transformation by electroporation.
   (i) Pre-chill 0.1 mm Biorad gene pulser cuvettes at −20 °C.
   (ii) Thaw *F. nucleatum* strain ATCC23726 competent cells on ice (~10 min).
   (iii) Prepare TSPC medium by mixing 10 mL TSP medium with 0.5 mL 1% L-cysteine and aliquot 1 mL into 1.5 mL Eppendorf tubes.
   (iv) Add 10 μL (~1 μg) pBCG02a-Δ*ftsX* plasmid DNA from Subheading 3.2 to 100 μL of competent cells and gently mix. Let it stay on ice for 10 min.
   (v) Transfer 100 μL of competent cells with plasmid into a cold cuvette.
   (vi) Electroporate the cells using the following conditions: 2.5 kV, 25 μF, 200 Ω in Gene Pulser Xcell modular electroporation systems.
   (vii) Quickly transfer the electroporated competent cells into 1 mL TSPC medium.
   (viii) Incubate cells in an anaerobic chamber at 37 °C for 3 h.
   (ix) Plate the transformed cells onto TSPC agar plates containing 5 μg/mL thiamphenicol.
   (x) Incubate the plates under anaerobic conditions at 37 °C for 3 days.
2. Pick up and inoculate three colonies resistant to thiamphenicol into a single 8 mL TSPC medium. Incubate under the anaerobic condition overnight at 37 °C.

3. Transfer 8 μL of overnight culture to fresh 8 mL TSPC broth without antibiotics and incubate overnight under anaerobic conditions at 37 °C (to allow the second crossover event to occur, resulting in the loss of the intergraded plasmid, *see* Fig. 1c, **step 2**).
4. Dilute the overnight culture 1000 and 10,000 times using fresh TSPC medium. Take 100 μL from the two diluted cultures and spread them on TSPC agar plates containing 2 mM theophylline. Then, incubate the plates under anaerobic conditions for 3 days at 37 °C.
5. Use sterile Polyester tipped swabs to pick 10–20 single theophylline-resistant colonies from **step 4** and streak onto TSPC plates and TSPC plates containing 5 μg/mL thiamphenicol to test for thiamphenicol sensitivity. Incubate for 3 days under anaerobic conditions at 37 °C.
6. By colony PCR, select 10 thiamphenicol-sensitive strains to screen for mutants lacking *ftsX* using primers P3 and P6. The results revealed that 5 of the 10 randomly chosen colonies were deletion mutants, while the other five reverted to the wild type during the second recombination event. Bands of approximately 2 kb indicate *ftsX* deletion, whereas bands of about 2.7 kb indicate a wild-type (WT) genotype (*see* Fig. 1d). Phase-contrast microscopy analysis was conducted to substantiate further that these mutants result from *ftsX* deletion. The observations demonstrated that the Δ*ftsX* strain's cells exhibited an elongated, filamented morphology, aligning with the *ftsX* mutant previously generated through a *galK*-based allelic exchange method [16] (*see* Fig. 1 e and the inserted graph).
7. The Δ*ftsX* clones are prepared for storage by inoculating individual colonies into 8 mL TSPC overnight under anaerobic conditions at 37 °C.
8. Prepare glycerol stock of Δ*ftsX* strain for −80 °C storage.
   (i) Grow individual mutant colonies in 8 mL TSPC medium in 16 mm culture tubes with caps. Transfer the culture to a 15 mL tube and spin at 6000 RPM for 5 min.
   (ii) Discard the supernatants and resuspend the cell pellets in a final volume of 600 μL of TSPC medium.
   (iii) Transfer the resuspended cultures to appropriately labeled cryogenic tubes. Add 400 μL of 25% glycerol and gently mix.
   (iv) Store the cryogenic tubes in the −80 °C freeze.

4 Notes

1. Stocks exposed to air for over 3 days should be discarded. A high concentration of L-cysteine, such as 5 g/L, is toxic to *F. nucleatum*.
2. Sterile-filter through a 0.22 μm filter using a 10 ml sterile syringe; make 1 mL aliquots in 1.5 mL sterile Eppendorf tubes and store at -20 °C until use.
3. All growth media should be pre-reduced in an anaerobic chamber for at least 24 hours before use.
4. Larger homologous flanking regions can significantly increase transformation efficiency. While mutating larger genes, using larger homologous flanking regions is recommended, and another set of mutant screening primers can be designed to produce 200-500 bp mutant bands.
5. pBCG02 can be directly used to generate the vector backbone for the gene deletion method as described article [21]. If pBCG02 is used as a template, the primers for PCR amplification of the plasmid backbone (removing *oriFn* and *repA*) are as follows:

   pCWU6-F: GTCCTAGAACTAGTGAAATTTCCG.

   pCWU6-R: GTAGCTTCCATACATATCACACTC.

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Chapter 7

Super-Resolution Microscopy of the Bacterial Cell Wall Labeled by Fluorescent D-Amino Acids

Chen Zhang and Suliana Manley

Abstract

Fluorescent D-amino acids (FDAAs) enable in situ visualization of bacterial cell wall synthesis via their incorporation into peptidoglycan (PG) crosslinks. When combined with super-resolution microscopy, FDAAs allow the details of cell wall synthesis to be resolved beyond the diffraction limit of visible light. Here, we describe using the super-resolution method of single-molecule localization microscopy (SMLM) in conjunction with two newly synthesized FDAAs (sCy_5DA and $sCy_5DL_{\_amide}$) to resolve bacterial PG at the nanoscale in a variety of species, including Gram-negative, Gram-positive, and mycobacteria.

Key words Fluorescent D-amino acids, Bacterial peptidoglycan, Super-resolution imaging, Single-molecule imaging

1 Introduction

Peptidoglycan (PG), a polymer composed of long glycan chains crosslinked by D-amino acids (DAA) to form a mesh-like network, is one of the main components of the bacterial cell envelope [1]. Through its elastic properties, the PG cell wall contributes to maintaining cell shape and size by resisting intracellular turgor pressure. During cell growth and division, cell shape changes occur via new PG synthesis and insertion into the existing cell wall, and old PG hydrolysis and release [2]. PG synthesis thus has attracted interest as an antibiotic target due to its role in cell cycle progression, and its exclusivity to bacteria. To investigate the dynamics of PG growth in vivo, fluorescent D-amino acids (FDAA) were developed by conjugating fluorophores with DAA backbones [3]. FDAAs enable nascent PG biosynthesis to be visualized in living cells by incorporating into PG cross-links via transpeptidase-mediated reactions [4]. Previous contributions to an FDAA toolkit (e.g., FDAAs with distinct fluorescence spectra

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and fluorogenic FDAAs) have enabled researchers to study mechanisms of cell wall remodeling in different bacterial species [5–8].

On the other hand, the small size of bacteria (typically 1–3 μm in length, 0.5–1 μm in width), which lies at the diffraction limit of conventional light microscopy (~250–400 nm), creates challenges to observing their PG ultrastructure. Single-molecule localization microscopy (SMLM, including PALM and STORM), which can provide spatial resolution far below the diffraction limit (~30 nm) [9], thus serves as a powerful tool to resolve detailed PG synthesis at the nanoscale. SMLM imaging relies on photoswitching: nonlinear optical transitions of the fluorophores that allow them to transition between fluorescent and dark states, to allow single molecules to be separated in time and space. Once each fluorophore is spatiotemporally isolated from its neighbors, one can reconstruct an SMLM image from localized single emitters, often captured over thousands of imaging frames [10]. The properties required of FDAAs for SMLM are particular: the probes must incorporate well into the bacterial cell wall, as well as photoblink efficiently. We have reported a set of FDAAs that enable visualization of PG by SMLM via one-step labeling [11]. These FDAAs further expand the repertoire of PG labeling tools and endow researchers with a nanoscale perspective on the spatial distribution of PG biosynthesis. In this chapter, we detail a protocol for culturing bacterial cells to label them with these SMLM-compatible FDAAs, and sample mounting for SMLM imaging.

2 Materials

Prepare all solutions using ultrapure water (18.2 MΩ-cm at 23 °C) and analytical grade reagents. All FDAAs mentioned here are commercially available from Tocris Bioscience. Carefully follow all regulations when disposing of waste materials and chemicals.

2.1 FDAAs

1. The far-red emitting FDAAs, sCy_5DA (729.26 g/mol) and $sCy_5DL_{\_amide}$ (770.33 g/mol), are used for SMLM imaging. Green FDAA, sBADA (594.25 g/mol) is used for wide-field (WF) imaging.
2. Dissolve FDAA powder into DMSO and adjust the volume to make a 20 mM stock solution. Make aliquots of 5 μL each in PCR tubes. Store at −20 °C (*see* **Note 1**).

2.2 dSTORM Buffer

1. 1 M MEA. Weigh 0.77 g cysteamine (MEA) and transfer to a 15 mL centrifuge tube containing 10 mL of PBS. Make aliquots of 100 μL each in 1.5 mL Eppendorf tubes. Store at −20 °C (*see* **Note 2**).

2. 200 mM sodium sulfate. Weigh 0.252 g sodium sulfate and transfer to a 15 mL centrifuge tube containing 10 mL of PBS. Store at 4 °C (*see* **Note 3**).
3. 1 mL 2× dSTORM buffer: combine 780 μL PBS, 20 μL 1 M MEA, and 200 μL 200 mM sodium sulfate in a 2 mL Eppendorf tube. Mix thoroughly by pipetting 3–5 times (*see* **Note 4**).

2.3 Agarose Pad

1. Gasket: press a clean silicone gasket (e.g., 15 mm in diameter and 1 mm in depth) onto the surface of a glass slide. Make sure the gasket is affixed to the glass slide without any visible bubbles in between (*see* **Note 5**).
2. 3% agarose solution: add 10 mL water to a 50 mL tube or beaker. Weigh and transfer 0.3 g low melting point ultrapure agarose into the water. Disperse the agarose powder thoroughly by vortexing. Loosen the tube cap and stand the tube upright in a 250 mL beaker. Heat the tube in a microwave (medium-high power, ~700 W). Stop the microwave every 5–10 seconds to observe whether agarose powders are completely dissolved (no visible agarose particles, uniform appearance of the solution). Heating the agarose solution for brief periods but multiple times will avoid boiling. Once the agarose is fully dissolved, place the tube into a water bath (50–55 °C) to avoid solidification (*see* **Note 6**).
3. Add 1 mL 3% agarose solution into a 2 mL Eppendorf tube containing 1 mL of 2× dSTORM buffer prepared before (*see* Subheading 2.2). Mix them by pipetting up and down 3–5 times while avoiding bubble formation (*see* **Note 7**).
4. Add an adequate volume of the mixture into the silicon gasket to fill it (e.g., 500 μL mixture for the gasket with the size mentioned above). Quickly seal the gasket from the top with another glass slide. Put the agarose pad into the fridge (4 °C) for ~20 min to allow it to fully gel (*see* Fig. 1a) (*see* **Note 8**).

3 Methods

3.1 Cell Culture and Labeling

Depending on the goal of experiments, one can label bacteria with FDAA with a long-pulse, short-pulse, or pulse-chase. In long-pulse labeling, incubate dyes with cell cultures for 1–3 cell cycle durations so that the whole bacterial outline can be visualized; for short-pulse labeling, incubate dyes with cells for 5–10% of the cell cycle so that nascent PG growth can be highlighted; for pulse-chase labeling, incubate cells with two (or multiple) FDAAs sequentially so that PG growth over different cell cycle stages can be tracked. Experimental details are illustrated below.

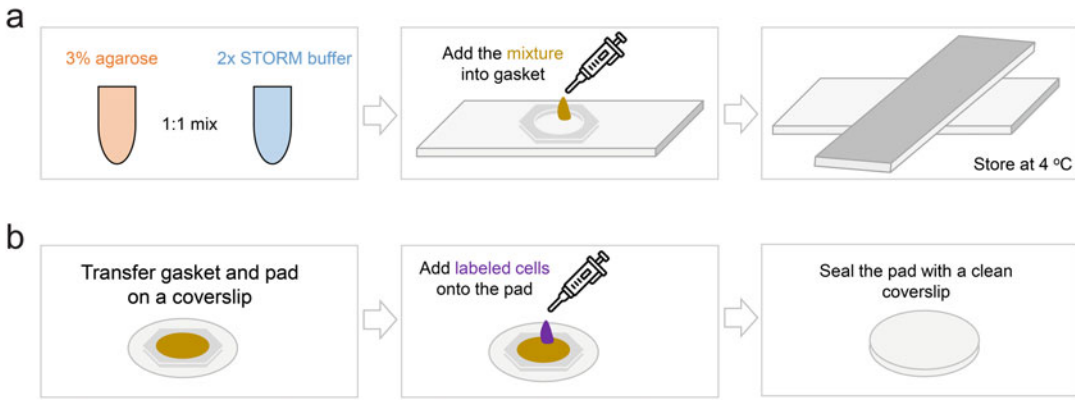

Fig. 1 Sample preparation by (**a**) making the agarose pad and (**b**) mounting the sample onto a coverslip for dSTORM imaging. Note that this illustration depicts 25 mm diameter coverslips which will be mounted into a special holder for SMLM imaging, thus both gasket and pad need to be transferred onto another coverslip. The transfer step can be skipped if the stage of microscope can directly hold a glass slide (*see* **step 2** in Subheading 3.2)

Table 1
Conditions of FDAA labeling tested in different bacteria species

Bacteria	Medium	Temp. (°C)	FDAA	Concentration (mM)
Caulobacter crescentus	PYE	30	sCy_5DA	0.5
Vibrio cholerae	M9	30	sCy_5DA	0.5
Klebsiella pneumoniae	M9	37	sCy_5DA	0.5
Pseudomonas aeruginosa	M9	37	sCy_5DA	1
Bacillus subtilis	LB	37	sCy_5DL, $sCy_5DL_{\_amide}$	0.1
Paraclostridium spp.	RCM	37	sCy_5DA	0.1
Staphylococcus aureus	LB	37	sCy_5DA	0.1
Streptococcus pneumoniae	C + Y	37	sCy_5DA	0.1
Mycobacteria smegmatis	7H9	37	sCy_5DA	0.1
Mycobacteria abssensus	7H9	37	sCy_5DA	0.5
Mycobacteria marinum	7H9	30	$sCy_5DL_{\_amide}$	1
Mycobacteria tuberculosis	7H9	37	$sCy_5DL_{\_amide}$	1

1. Inoculate a single colony of bacteria from an agar plate into a Falcon tube containing 3 mL culture medium and grow cells overnight. Use a culture medium, temperature, and shaking speed suitable for the chosen species (*see* Table 1).
2. Measure the optical density (OD_{600}) of grown cell cultures by nanodrop or plate reader. Dilute cell cultures into 3 mL fresh medium for an initial OD_{600} 0.05–0.1. For instance, add

200 μL cell culture (OD_{600} ~ 1.5) into 2.8 mL medium to reach an initial OD_{600} ~ 0.1.

3. For long-pulse labeling, add 15 μL of FDAA stock solution directly into 3 mL of cell culture (final concentration of 0.1 mM), and grow cells with dye incubation for 1–3 cell cycle durations (e.g., 20–60 min for *Bacillus subtilis* cultured in LB). The optimal concentration of FDAA may vary between bacterial species and growth conditions (*see* Table 1) (*see* **Note 9**).
4. For short-pulse labeling, grow cells from **step 2** until they reach mid-exponential phase (e.g., OD_{600} 0.3–0.5 for *Caulobacter crescentus*). Add 15 μL of FDAA stock solution into the 3 mL cell culture (final concentration of 0.1 mM), and then grow cells for 5–10% cell cycle duration (e.g., ~5 min for *C. crescentus* cultured in PYE) (*see* **Note 10**).
5. For pulse-chase labeling (using *C. crescentus* as an example here), grow and label cells using 15 μL of sBADA (final concentration of 0.1 mM, as described in **step 3** or **step 4**); wash cells three times by centrifugation (10,000 g, 1 min) and resuspend in 1 mL of fresh medium; then add 25 μL of sCy_5DA (final concentration of 0.5 mM) for a short-pulse labeling (as described in **step 5**) (*see* **Note 11**).

3.2 Cell Fixation

1. Collect 3 mL of labeled cell culture (from **steps 4**, **5**, or **6** in Subheading 3.1) into 2 mL Eppendorf tube(s).
2. Wash cells three times by centrifugation (10,000 g, 1 min) and resuspend in 1 mL of PBS (*see* **Note 12**).
3. Resuspend cell pellets in 500 μL of 4% paraformaldehyde (PFA) in PBS, in hood (*see* **Note 13**).
4. Leave the fixed cells in a drawer (to avoid light) for 10 min at the room temperature.
5. Collect cell pellets by centrifugation (10,000 g, 1 min), and discard the PFA supernatant.
6. Wash cells again by resuspending pellets in 500 μL of PBS, followed by centrifugation (10,000 g, 1 min) (*see* **Note 14**).
7. Resuspend cells into 20 μL of 1× dSTORM buffer (made by 1:1 volumetric ratio of mixing 2× dSTORM buffer and PBS). Adjust the volume by adding more 1× dSTORM buffer to obtain a final OD_{600} ~ 0.5 (*see* **Note 15**).

3.3 Sample Mounting

Depending on the sample holder, the sample mounting may need to be adapted. We describe two approaches here.

1. Carefully remove the top slide from the agarose pad by sliding it to the side rather than lifting upward. Inspect the surface of pad to ensure that it is flat and clean (*see* **Note 16**).
2. If the microscope stage can hold a rectangular glass slide, one can skip this step and directly add cells to the pad (*see* **Note 17**). In the case of a holder designed for 25 mm coverslips, carefully detach the gasket from the bottom slide and the pad, lifting it upward using tweezers. Remove any excess agarose from the gasket. Slide the agarose pad from the glass slide into the center of a coverslip (25 mm in diameter) by pushing it gently with tweezers. Encircle the pad with the cleaned gasket and press the gasket until it firmly attaches to the coverslip surface (*see* Fig. 1b).
3. Spot 2–3 μL cell suspension (from "Cell fixation" **step 7**, in 1× dSTORM buffer) onto the agarose pad. Spread the drop as uniformly as possible by tilting and rotating the coverslip.
4. Cover the agarose pad with foil (to avoid light exposure) and leave it in the hood. Check every 2 min until the cell suspension is fully absorbed into the pad (*see* **Note 18**).
5. Seal the agarose pad with a plasma cleaned coverslip (*see* **Note 19**). In the case of a round holder for 25 mm coverslips, the pad and gasket are now sandwiched between two 25 mm coverslips and placed into the holder.

3.4 SMLM Imaging and Reconstruction

1. For long-pulse labeling using sCy_5DA or $sCy_5DL_{\_amide}$, first capture a WF image (200 ms exposure, 6.8 W/cm^2). Cell outlines should be fully labeled, and the labeling on different cells should show similar intensity profiles. Then start SMLM imaging (10 ms exposure, 3.4 kW/cm^2, 20,000 frames) using the 642 nm laser line (*see* **Note 20**).
2. For pulse-chase labeling using sBADA and sCy_5DA in *C. crescentus*, first capture a WF image of sBADA labeling (200 ms exposure, 2.1 W/cm^2) using the 488 nm laser line, and acquire the WF and SMLM images of sCy_5DA (as described in **step 1**).
3. Switch on the 405 nm laser line manually (beginning from minimum power) when molecules become too sparse [12]. Gradually increase the 405 nm laser power to maintain the density of single molecules during the acquisition process (*see* **Note 21**).
4. Super-resolution images can be reconstructed and visualized with the ThunderSTORM plugin in ImageJ [13, 14] (*see* **Note 22**). As results of WF and SMLM imaging of long-pulse labeled bacteria show, the sCy_5DA or $sCy_5DL_{\_amide}$ perform well in almost all tested species (*see* Fig. 2). Specifically, some morphological features not visible in widefield images (e.g., the

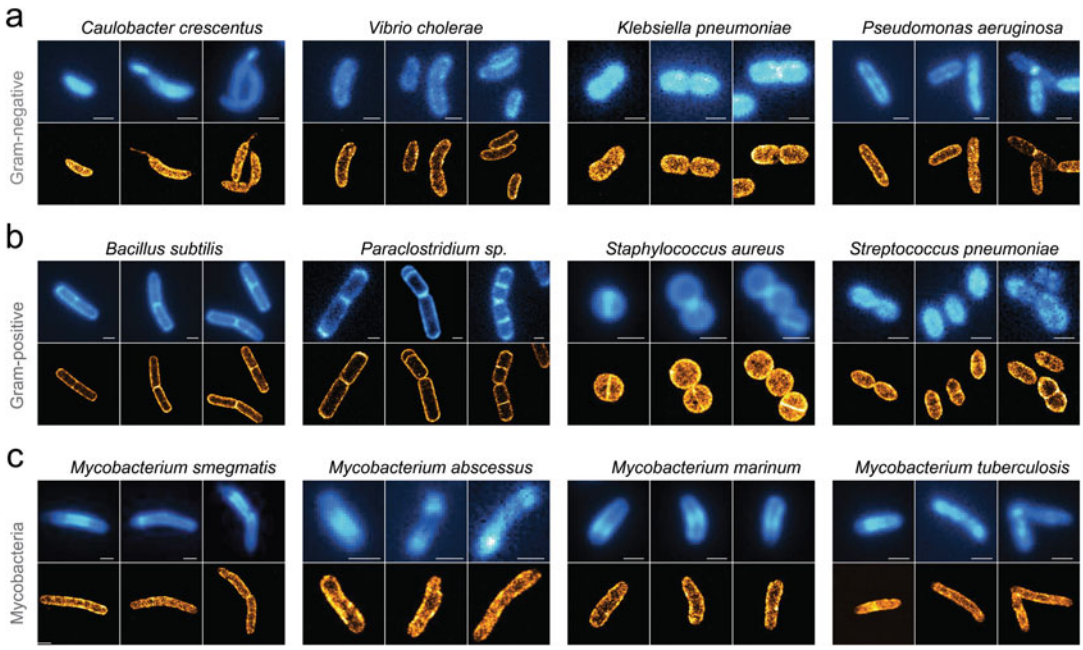

Fig. 2 WF and SMLM imaging of (**a**) Gram-negative bacteria, (**b**) Gram-positive bacteria, and (**c**) mycobacteria species. Conditions for cell growth and labeling are listed in Table 1. Scale bar: 1 μm. (Reprinted with permission from ref. 11. Copyright 2022 American Chemical Society)

equatorial ring of *S. aureus*) can be clearly visualized and quantified under SMLM. Pulse-chase labeling of *C. crescentus* via sBADA and sCy_5DA revealed its nascent PG growth patterns with nanoscale details, for instance, the asymmetric polar or mid-cell zonal PG biosynthesis, and the double-ring structure or small cracks at the mid-cell region (*see* Fig. 3).

5. The quality of SMLM images can be further evaluated by measuring the distance between distinguishable objects or by the Fourier-ring correlation method [15]. Empirically, a well-optimized SMLM image of bacterial populations should resolve neighboring cells that are ~100–200 nm apart and give an FRC resolution ~50–90 nm (*see* **Note 23**).

4 Notes

1. Avoid refreezing FDAA aliquots. The sCy_5DA dye is superior to label Gram-negative bacteria while $sCy_5DL_{\_amide}$ labels some Gram-positive and mycobacteria species well.
2. The stored MEA aliquots can be used for ~6 months. Avoid refreezing MEA aliquots. The MEA stock (powder) inadvertently rehydrates and oxidizes once opened. Order and use a fresh MEA stock if powders become clumpy or gel-like.

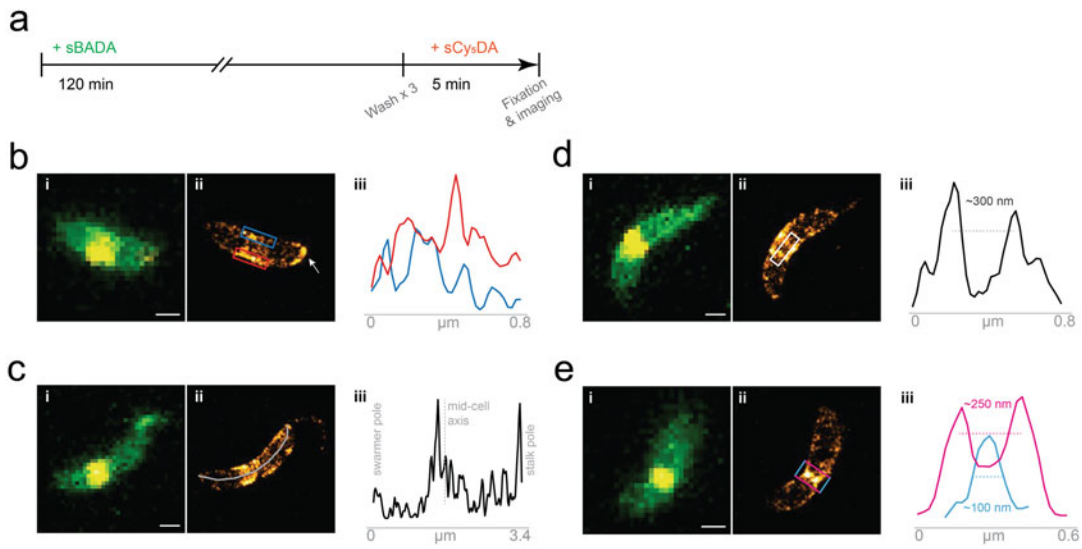

Fig. 3 (**a**) Schematic of the pulse-chase labeling strategy: *C. crescentus* cultured in PYE was incubated with 0.25 mM sBADA for 120 min, then with 0.5 mM sCy_5DA for 5 min. (**b**–**d**) (i) WF images of pulse-chase labeled *C. crescentus* cells, sBADA (green) and sCy_5DA (yellow); (ii) SMLM imaging of sCy_5DA; (iii) intensity profile along the long axis of rectangle (**b** and **d**), the midline (**c**), or two axes of the rectangle (**e**). Scale bar: 500 nm. (Adapted with permission from ref. [11]. Copyright 2022 American Chemical Society)

3. The sulfite sodium buffer can be used for ~2 weeks. Keep it at 4 °C for reuse. Seal the lid of sulfite sodium stock using parafilm since it is moisture sensitive.
4. Different dSTORM buffer recipes are worth trying when optimizing the imaging quality [16–18]. In preliminary experiments, adding 50 mM (final concentration) of β-mercaptoethanol (BME) to the current 1× dSTORM recipe could improve photoblinking.
5. After making pads there would be dry agarose patches sticking on the surface of gaskets. To remove them, rinse gaskets with 100% acetone in a 50 mL tube, followed by sonication for 20 min. After cleaning, rinse gaskets in H_2O and keep them at room temperature. Dry gaskets by KimTech paper before use. Glass slides may be cleaned in ethanol followed by air drying. Do not reuse glass slides after making pads since sticky agarose patches are difficult to remove completely.
6. Always make fresh agarose solution on the day of imaging. Avoid reusing (re-heating) the solidified agarose since the concentration may change during re-heating cycles.
7. Perform this step quickly to avoid agarose solution solidifying before adding it into gasket well. Otherwise, small agarose clumps might form, and the resultant agarose pad will not have a flat surface.

8. Use the agarose pad within ~6 hours after making it. The efficacy of dSTORM buffer within the pad may decay over time. Also, excess agarose gel around the gasket can dehydrate in the fridge after storage, which would make it difficult to remove the upper slide smoothly.
9. Notably, 0.1 mM is a good concentration to start with, previous studies suggested that a higher concentration of 0.5–1 mM would not impact cell growth and morphology [5].
10. Cells at exponential phase generally have optimal labeling results. To minimize the dye usage while maintaining a high incubation concentration, one could first collect cell pellets by centrifugation (10,000 g, 1 min) and resuspend into 100 μL fresh medium in a 1.5 mL Eppendorf tube, then add 5 μL dye for short-pulse labeling. For some bacteria that can easily form aggregates (e.g., Mycobacteria), filter the cell culture with a 5 μm pore filter to remove cell clumps before adding the dye for short pulse labeling.
11. After multiple washing steps, the number of cells in the pellets may diminish. If that happens noticeably, use a higher centrifugation speed (e.g., 15,000 g) or begin with more cells.
12. The cells/pellets are best centrifugated/kept at 4 °C and resuspended with cold PBS to slow bacterial cell wall growth. The total duration of washing steps (~5 min) may lead to diminished labeling patterns for some fast-growing cells (e.g., *E. coli* and *B. subtilis*).
13. Different fixation methods (e.g., PFA, methanol, or cryofixation) can be tested and optimized when imaging FDAA together with other fluorescent labeling (e.g., fluorescent proteins). PFA preserves bacterial morphology well but may cause artifacts of intracellular protein structures [19].
14. The number of cells may diminish after multiple washing steps. If the pellet is no longer visible, one should grow and stain a larger volume of cell culture from the beginning. After fixation, cells can be further stained with other dyes (e.g., DAPI, SYTOX green). Using another fluorescent dye can help to find cells in wide-field and estimate the focal plane from another channel, which will minimize the photobleaching of sCy_5DA or $sCy_5DL_{\_amide}$.
15. Depends on the number of cells remained, the amount of 1× dSTORM buffer to resuspend cells may vary and 20 μL is a good volume to start with. The OD_{600} measured here only gives a proxy of cell density and based on that, one can further estimate the volume of cell suspensions to be spotted on the agarose pad. This is practically useful since one needs a moderate cell density in a field-of-view (FOV) for image acquisition. When cells are too dense, it can be difficult to segment cells for

analysis; if too sparse, it reduces the acquisition efficiency. Empirically, spotting 2–3 μL of *C. crescentus* resuspension (OD_{600} ~ 0.5) gives a good cell density on agarose pads.

16. Re-make agarose pad if the surface looks wrinkled. A poor-quality agarose pad will lead to cells clumping and appearing to lie in different focal planes.
17. Before performing this step, prepare the sample (cell suspension in 1× dSTORM buffer, from **step 7** in Subheading 3.2) ready-for-use. Leave the agarose pad in air too long will lead to its evaporation and shrinkage.
18. This step is very critical, and one should be very patient! Any visible liquid left on the pad before sealing would lead to cell "swimming" inside. However, over drying will shrink pads, thus the surface of pad will become lower than the gasket and prevent it from attaching to the sealing coverslip.
19. Coverslips were first air blown to remove any large visible particles and put onto a Teflon rack (holding 10 coverslips), and placed in the chamber of a plasma cleaning device. A cleaning time of 3 min is applied. Notably, clean coverslips are significantly important to avoid artifacts in single-molecule imaging, and plasma cleaning is suggested to be a better tool compared with other cleaning methods (e.g., ethanol, KOH + UV) [20].
20. Parameter settings and acquisition steps are controlled and implemented by a Micro-Manager script on a custom-built SMLM microscope [21], which may vary from a commercial microscope. However, a WF image acquisition is always recommended for the comparison with reconstructed super-resolution images and can be useful for potential troubleshooting.
21. Empirically, the 405 nm laser line can be turned on when ~10,000 frames are acquired. Without enough photoblinking, the SMLM image may display an intensity profile inconsistent with the WF image.
22. ThunderSTORM is a free and well-documented ImageJ plugin for SMLM data processing and analysis. Alternatively, there are a few published software that work on similar functions [22, 23].
23. To perform FRC measurements, single-molecule localizations from odd and even frames are first separated to render two images, and they are used as inputs to generate the FRC curve (by ImageJ plugin: BIOP- > Image Analysis- > FRC- > FRC Calculation, details can be found online: https://github.com/BIOP/ijp-frc). Finally, a fixed threshold of 1/7 (~0.143) is

chosen as a threshold of FRC value for the resolution determination.

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Chapter 8

Type I Lipoteichoic Acid (LTA) Detection by Western Blot

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Abstract

Type I lipoteichoic acid (LTA) is a glycerol phosphate polymer found in the cell envelope of diverse Gram-positive bacteria including *Staphylococcus aureus, Bacillus subtilis,* and *Listeria monocytogenes*. The polymer is linked by a lipid anchor to the outer leaflet of the bacterial membrane and in some bacteria can also be shed and detected in the culture supernatant. Here, we describe a simple and rapid western blot method for the detection of Type I LTA in bacterial cell extracts and culture supernatant fractions using a polyglycerol phosphate specific monoclonal LTA antibody.

Key words Western blot, Type I LTA, Monoclonal polyglycerol phosphate specific LTA antibody, Bacterial cell extracts, Culture supernatant samples, SDS-PAGE

1 Introduction

Western blot analysis is typically used to detect specific proteins in cell extracts. For this method, which was first described in 1979, cell extracts are prepared, proteins separated by size by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), electro-transferred to nitrocellulose or polyvinylidene difluoride (PVDF) membranes, and finally the protein of interested is detected using a protein-specific antibody [1–3]. Type I LTA is a polyglycerol phosphate cell wall polymer produced by many Gram-positive bacteria belonging to the Bacillota phylum. We found that Type I LTA can also be detected by western blot using bacterial cell extracts or supernatant fractions and a polyglycerol phosphate specific primary antibody [4, 5]. In this protocol, methods are described for the growth and preparation of bacterial cell extracts and culture supernatant samples for the detection of Type I LTA by western blot. The method will be described for the preparation of *Staphylococcus aureus* extracts but can be easily adapted for the detection of type I LTA in other Gram-positive bacteria [6, 7]. Using this method some information can also be gained on the amount of LTA produced by different strains. Furthermore,

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a difference in the LTA migration behavior in gels between samples derived from wild-type or mutant strains is indicative of structural LTA alterations [4, 6–8]. However, to obtain more detailed information on the chemical structure of LTA, the cell wall polymer needs to be purified and a more detailed structural analysis performed [9–13]. A method for the purification and nuclear magnetic resonance (NMR) analysis of Type I LTA is described in another protocol in this issue.

2 Materials

All solutions should be prepared using ultrapure water (deionized water further purified to obtain a resistivity of 18.2 MΩ-cm). The reagents are stored at room temperature unless otherwise specified. Some reagents will need to be handled using appropriate protective equipment and disposed of through dedicated waste routes, and for this country and institution specific regulations and procedures should be followed.

2.1 Bacterial Growth

1. Bacterial culture tubes (e.g., 30 mL universal tubes).
2. Tryptic Soy Broth: 30 g per L dissolved in water and sterilized by autoclaving (*see* **Note 1**).
3. Shaking incubator set to 37 °C (*see* **Note 1**).

2.2 Gel Electrophoresis

1. Small beakers.
2. Resolving gel buffer: For 500 mL: 90.9 g Tris, 20 mL 3 M NaCl, 8 mL 0.5 M EDTA pH 8, 20 mL 10% SDS; adjust pH to 8.8 with concentrated HCl.
3. Stacking gel buffer: For 500 mL: 250 mL 1 M Tris pH 6.8, 20 mL 3 M NaCl, 8 mL 0.5 M EDTA pH 8, 20 mL 10% SDS.
4. 30% Acrylamide:bis-acrylamide solution (29:1); store at 4 °C (*see* **Note 2**).
5. Ammonium persulfate: 10% solution in water, store the solution at 4 °C for up to three months.
6. Tetramethylethylenediamine (TEMED), store at room temperature.
7. Optional: 10% w/v bromophenol blue solution.
8. SDS-PAGE gel running buffer (10× stock): Dissolve 144 g Glycine, 30 g Tris, 10 g SDS in a final volume of 1 L ddH_2O; for use dilute to 1× with ddH_2O.
9. Pre-stained protein marker covering the 10 kDa to 250 kDa range.

10. Mini Gel Caster system (e.g., Hofer 4 to 5 gel caster system SE275).
11. Vertical Mini gel electrophoresis system including plates, 0.75 mm spacers and 10 or 15 well combes (e.g., Hofer Mini Vertical protein Electrophoresis Unit SE250).
12. Power pack.

2.3 Cell Lysis and Sample Preparation

1. 2 mL screwcap tubes.
2. 1.5 mL microcentrifuge tubes.
3. 0.1 mm glass beads.
4. Vortex mixer.
5. Vortex adaptor to hold microcentrifuge tubes.
6. SDS-PAGE sample buffer (1×): 0.0625 M Tris pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 10% ß-mercaptoethanol (*see* **Note 3**).
7. Cold room.
8. Heat block set to 100 °C.

2.4 Electrophoretic Transfer

1. Transfer buffer: 2 L ddH_2O, 0.5 L Methanol, 7.5 g Tris, 36.25 g Glycine.
2. Tris buffered saline (TSB) (10× solution): 2.4 L ddH_2O, 60.5 g Tris, 200 g NaCl, ~30 mL conc. HCl – adjust pH to 7.6, Fill up to 2.5 L with ddH_2O and autoclave.
3. Tris buffered saline with Tween 20 (TSBT) (1× solution): 250 mL 10× TBS, 2250 mL ddH_2O, 2.5 mL Tween 20.
4. Polyvinylidene fluoride (PVDF) membrane cut to size of gel.
5. Whatmann paper cut to size of transfer cassette.
6. 100% Methanol.
7. Wet tank transfer system (e.g., Hofer Transfer tank TE42).
8. High current power supply.
9. Plastic containers of different sizes (e.g., empty tip boxes, Tupperware boxes).

2.5 Detection of Type I LTA

1. 5% dry milk in 1× TBST: Dissolve 5% w/v dry milk powder in 1 × TBST and store up to 3 days at 4 °C.
2. 5 mg/mL human IgG (*see* **Note 4**).
3. Primary antibody: Lipoteichoic acid monoclonal antibody (mAB) Clone 55 (Hycult Biotech product code HM2048-200UG) (*see* **Note 5**).
4. Secondary antibody: Anti-mouse IgG, HRP-linked antibody.
5. ECL detection reagent (e.g., Clarity Western ECL substrate from Bio-Rad).

6. Plastic container large enough to accommodate PVDF membrane (e.g., empty tip box).
7. Rocking shaker at room temperature and at 4 °C.
8. Clear plastic sheets.
9. Western blot Imaging system (e.g., Bio-Rad ChemiDoc Touch Imaging System).

3 Methods

3.1 Bacterial Growth for LTA Western Blot Analysis

1. Prepare a 30 mL culture tube with 3–5 mL of TSB medium and add any antibiotics or inducers as required (*see* **Note 1**).
2. Inoculate the tube with a single colony of a *S. aureus* strain or bacterial strain of interest (*see* **Note 1**).
3. Incubate the tube overnight at 37 °C with shaking at 180 rpm (*see* **Note 6**).

3.2 Cell Lysis and Sample Preparation for LTA Western Blot Analysis

1. Using an aliquot of the culture, measure the absorbance of the culture at 600 nm (A_{600}), which is needed for normalization purposes between samples, described in **step 11** and optional **step 21** in this section (*see* **Note 7**).
2. For the preparation of cell extract samples, add the equivalent of a 0.5 mL volume of 0.1 mm glass beads to a 2 mL screwcap tube.
3. Add 1 mL of the culture to the tube with the glass beads.
4. Tighten the lid and place the tubes in the vortex adaptor taking care to balance the tubes on opposite sides of the adaptor.
5. Vortex vigorously for 45 min at 4 °C.
6. Remove the tube from the vortex adaptor and vortex the tube briefly in the upright position to remove any glass beads that may have accumulated in the lid of the tube.
7. Centrifuge the samples at 200 ×*g* for 1 min at room temperature to settle the beads to the bottom of the tube. At this low speed only the glass beads will pellet but not the lysed cells containing the membrane and LTA.
8. Remove 500 μL of the supernatant containing the cell debris without disturbing the glass beads and transfer it to a 1.5 mL microcentrifuge tube or 2 mL screwcap tube.
9. Centrifuge the sample with the lysed cells at 17 000 × *g* for 10–15 min at room temperature to pellet the bacterial debris.
10. Remove the supernatant completely with a pipette and discard.
11. Resuspend the pellet in 1× SDS-PAGE sample buffer. Calculate the volume of 1× SDS-PAGE sample buffer to use with the

following formula. Volume of 1× SDS-PAGE buffer = optical density of the culture x 15. For example, a culture with an OD of 6 will be resuspended in 90 μL of 1× SDS sample buffer.

12. Boil the sample in a heat block set to 100 °C for 20 min (*see* **Note 8**).
13. Centrifuge the sample at 17 000 ×*g* for 5 min at room temperature.
14. The cell extract sample can either be loaded straight away onto a polyacrylamide gel or stored frozen for several weeks at −20 °C for later use (*see* **Note 9**).

Additional optional steps for the preparation of culture supernatant samples.

15. Add 500 μL of the culture to a 1.5 microcentrifuge tube.
16. Centrifuge the sample at 17 000 ×*g* for 10 min at room temperature to pellet the bacteria.
17. Transfer 100–200 μL of the supernatant containing any released LTA into a clean 1.5 mL microcentrifuge tube or 2 mL screwcap tube (*see* **Note 10**).
18. Add an equal volume of 1× SDS sample buffer to the supernatant fraction.
19. Boil the samples in a heat block set to 100 °C for 20 min (*see* **Note 8**).
20. Centrifuge the sample at 17 000 ×*g* for 5 min.
21. The supernatant sample can either be loaded straight away onto a polyacrylamide gel or stored frozen for several weeks at −20 °C for later use (*see* **Note 9**). For normalization between samples, different volumes of the supernatant sample fraction can be loaded on a gel, based on the absorbance reading obtained in **step 1** of this section (Subheading 3.2). For example, if for a supernatant sample derived from a culture with an A_{600} of 6, 10 μL are loaded on a gel, only 8.6 μL are loaded on a gel from a sample obtained from a culture with an A_{600} of 7.

3.3 Gel Electrophoresis of LTA Samples

1. Prepare a standard 15% Tris-Glycine Mini SDS-PAGE gel (ca 10 cm wide, 8 cm high, and 0.75 mm thick) with 10 or 15 wells. Details for the preparation of 5 × 15% SDS-PAGE gels using a Hoefer mini 4 to 5 gel caster system SE275 are presented below but the protocol can be adjusted depending on the gel caster and electrophoresis system available in the laboratory.
2. Assemble the plates for 5 gels using 0.75 mm spacers in the gel caster as per the manufacturer's instructions.

3. Prepare the resolving gel by combining 6.9 mL ddH_2O, 7.8 mL resolving buffer, 15 mL of a 30% acrylamide:bis-acrylamide solution (29:1) and 300 μL APS (10% w/v) in a small beaker (*see* **Note 3**).
4. Add 30 μL TEMED, mix briefly and immediately pipet 23 mL of the mixture into the assembled Hofer 5 gel caster system SE275 (*see* **Note 11**).
5. Overlay each gel with ca. 1 mL of isopropanol and slide the combes between the plates (*see* **Notes 12–13**).
6. Let the gels polymerize at room temperature for 30–60 min (*see* **Note 14**).
7. Once polymerized, pour away the isopropanol and rinse the top of the gels 3–5 times with water. After the final rinsing step remove all water and wick away any remaining water drops with a paper towel and immediately proceed to the next steps.
8. Prepare the stacking gel mixture by combining 8.3 mL ddH_2O, 3.75 mL stacking buffer, 2.8 mL 30% acrylamide and bis-acrylamide solution (29:1) and 150 μL APS in beaker. Optionally add 3–5 μL of 10% w/v bromophenol blue solution (*see* **Note 15**).
9. Add 15 μL TEMED, mix briefly and overlay each gel in the caster with approximately 2–2.5 mL of the stacking mixture and immediately insert the combs.
10. Let the stacking gel polymerize at room temperature for approximately 60 min. Once polymerized remove the gels from the gel caster (*see* **Notes 14** and **16**).
11. Shortly before use of the gel, remove the comb and rinse the wells 2 or 3 times with water to clean the wells.
12. Assemble the 15% SDS-PAGE gel into the vertical electrophoresis tank unit (e.g., Hofer Mini Vertical protein Electrophoresis Unit SE250 or a similar system).
13. Add the 1× SDS running buffer to the upper and lower chambers so that the top and the bottom of the gel are covered with buffer and ensuring there are no leaks.
14. Load the LTA cell extract samples obtained in **step 14**, Subheading 3.2 into wells of the 15% SDS-PAGE gel and in one well the pre-stained protein marker (*see* **Note 17**).
15. (Optional) On a separate gel, load the supernatant sample obtained in **step 21**, Subheading 3.2 (*see* **Note 10**).
16. Electrophorese the gel at a constant voltage of 200 V for approximately 45 min until the dye front reaches the bottom of the gel.
17. Remove the gel from the tank and immediately proceed with the sample transfer to a PVDF membrane.

3.4 Electrophoretic Transfer of the LTA Sample to a PVDF Membrane

1. Fill the tank transfer system with transfer buffer. The protocol below is provided for a Hoefer wet transfer tank system TE 42 and can be adjusted depending on which wet tank system is available in the laboratory (*see* **Note 18**).
2. Prepare a PVDF membrane that is slightly larger than the resolving part of the gel (ca. 7 × 9 cm).
3. Wet the PVDF membrane in 100% methanol for 30 seconds and subsequently place the membrane in a small container with transfer buffer.
4. Place the sponges from the transfer cassette into transfer buffer.
5. Prepare 2 pieces of whatmann paper cut to the size of the transfer cassette and shortly before use, wet them with transfer buffer.
6. Using the gel from **step 17** Subheading 3.3, ease the plates apart using one of the spacers and remove one of the plates to release the gel.
7. Using one of the spacers, cut off the stacking gel and discard. Then gently lift the resolving gel part off the plate taking care not to break it and transfer the gel into a small container with transfer buffer.
8. Assemble the transfer cassette. Place one of the sponges on the open cassette, lay one of the whatmann filter papers on top. Place the PVDF membrane on the whatmann paper. Add the gel on top of the PVDF membrane, taking care that there are no bubbles between the PVDF membrane and the gel as this will affect the transfer. Place the second whatmann paper over the gel. And finally place the second sponge on top. Close the cassette and make sure to remember the side that the PVDF membrane is on. The flow of samples goes from the negative electrode (usually marked in black) to the positive electrode (usually marked in red). Therefore, the PVDF membrane must be closer to the positive electrode and the gel closer to the negative electrode.
9. Place the cassette in the transfer tank and apply a constant current. When using a Hoefer wet transfer tank system TE 42, the electro transfer is performed at 1000 mA for 1 hour. However, the transfer current and time will need to be adjusted depending on the tank transfer system used.

3.5 Detection of Type I LTA by Western Blot

1. Stop the transfer and remove the cassette from the transfer tank.
2. Lay the cassette on its side and open. Carefully peel away the layers of sponge and whatmann paper.
3. Remove the PVDF membrane and place it with the side that was facing the gel upward in a small plastic container.

4. Add 20 mL of 5% milk TBST blocking buffer. When working with *S. aureus* samples, also add 30 µL of 5 mg/mL human IgG as part of the blocking solution (*see* **Notes 4** and **19**).
5. Incubate the membrane in the blocking solution for 1 hour with shaking at room temperature (*see* **Note 20**).
6. Pour away the blocking solution and discard.
7. Add 20 mL of 5% milk TBST buffer and 30 µL of 5 mg/mL of human IgG.
8. Add 5 µL of monoclonal polyglycerol phosphate LTA Clone 55 primary antibody (1:4000 dilution) and incubate overnight with shaking at 4 °C (*see* **Note 21**).
9. Pour away the primary antibody solution and discard.
10. Wash the PVDF membrane once briefly with 20–40 mL of TBST. To do this add the TBST to the box, shake by hand and then discard the solution.
11. Wash the PVDF membrane 3 times for 10 min with 20–40 mL of TBST. To do this add 20–40 mL of TBST to the box containing the membrane and incubate with shaking at room temperature for 10 min, and afterwards discard the liquid. Repeat this step two more times to give a total of 3 washes of 10 min.
12. Add 20 mL of 5% milk TBST buffer, 30 µL of 5 mg/mL of human IgG.
13. Add 2 µL of the anti-mouse IgG, HRP-linked secondary antibody (1:10000 dilution) and incubate with shaking at room temperature for 2 h (*see* **Note 21**).
14. Pour away the solution and discard.
15. Wash the PVDF membrane once briefly with 20–40 mL of TBST. To do this add the TBST to the box, shake by hand and then discard the solution.
16. Wash the PVDF membrane 3 times for 10 min with 20–40 mL of TBST. To do this, add 20–40 mL of TBST to the box containing the membrane and incubate with shaking at room temperature for 10 min, and afterward discard the liquid. Repeat this step two more times to give a total of 3 washes of 10 min.
17. Prepare the Enhanced Chemiluminescence (ECL) reagent right before use by mixing 500 µL of each component. A total of 1 mL ECL solution is needed per membrane when using the ECL reagents as specified in the material list.
18. Place the membrane on a clear and colorless plastic sheet and apply the 1 mL of ECL reagent evenly across the membrane. Place another sheet of plastic on top of the membrane (*see* **Notes 22–23**).

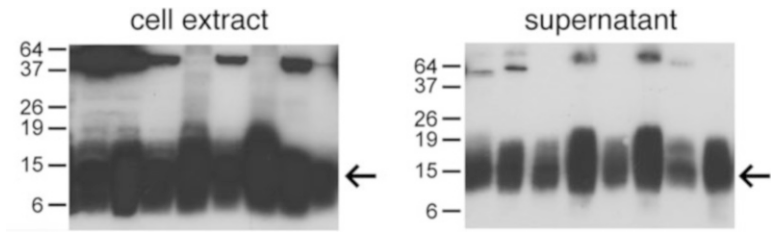

Fig. 1 Example of an LTA western blot result. Cell extract (left panel) and supernatant (right panel) samples were prepared from overnight cultures of different clinical *S. aureus* stains and separated on a 15% SDS-PAA gel as described in this protocol. The type I LTA was detected by western blot using the monoclonal polyglycerol phosphate specific LTA Clone 55 antibody. The region of the LTA-specific signal is indicated by an arrow on the right of each panel and the positions of protein molecular mass markers (in kDa) run alongside the samples are indicated on the left of each panel

19. Image the blot using a chemiluminescence imager such as a Bio-Rad Chemidoc Touch system. An example image of a western blot result using cell extract and supernatant samples derived from different *S. aureus* stains is shown in Fig. 1.

4 Notes

1. The protocol is described for the detection of type I LTA from *Staphylococcus aureus*, which is typically grown in TSB medium at 37 °C. However, the growth temperature can be varied, and the TSB medium can be substituted with other growth media. The method can also be applied to other Gram-positive bacteria producing type I LTA. For instance, we have used Lysogenic Broth (LB), LB with 0.1% glucose (LB-glucose) or Difco Antibiotic Medium 3 (PAB medium) for the growth of *Bacillus subtilis* strains or Brain Heart Infusion (BHI) medium for the growth of *Listeria monocytogenes* strains, and subsequently prepared samples for the detection of type I LTA.
2. Unpolymerized acrylamide is a neurotoxin and should be handled with care.
3. ß -mercaptoethanol should be added fresh to the sample buffer under a chemical hood and used within two weeks following the addition of ß-mercaptoethanol.
4. Human IgG is often used as additional blocking reagents when performing western blots with *S. aureus* extracts. Most *S. aureus* strains produce IgG-binding proteins, which would otherwise titrate away antibodies and give a very strong background signal on western blots. Human IgG can be omitted during the western blot procedure when working with bacteria

that do not produce IgG-binding proteins such as *B. subtilis* or *L. monocytogenes*.

5. We have noted some batch-to-batch variation in signal strength using this antibody. If no signal is obtained it is advisable to try a different batch. We also found that the antibody is stable for several years when stored frozen at −80 °C.
6. In place of using overnight cultures, the cultures can also be back-diluted the next day and grown to exponential phase and exponential phase cultures can be used to prepare extracts for the detection of type I LTA by western blot. Bacterial cultures can also be incubated at different temperatures.
7. Bacterial overnight cultures usually reach an absorbance above 1 and to determine the absorbance of the culture more accurately, the culture should be diluted in growth medium. Culture absorbance readings are no longer linear at high values, e.g., above 1. For *S. aureus* cultures we usually use a 1:10 or 1:20 dilution so that the absorbance reading is below 0.7. To calculate the actual absorbance of a sample, the measured absorbance of the diluted aliquot is multiplied by the dilution factor.
8. When using 1.5 mL microfuge tubes, it can help to put an extra heat block insert on top of the tubes to prevent the lids from opening during this heating step. Alternatively, a small hole can be made in the lid of the tube to prevent the lid from opening, however some of the liquid might evaporate in this case. Also, for some bacterial species a better result is obtained when the heating step is extended from 15–20 min to 45 min.
9. When samples are stored frozen, they should be heated once more for 3–5 min at 100 °C and subsequently centrifuged at 17000 ×*g* g for 5 min at room temperature right before loading them on the polyacrylamide gel.
10. The supernatant fraction will contain any LTA released from the membrane of bacterial cells and will likely also contain LTA derived from bacterial cells that have lysed during bacterial growth. There is a great variation in how much LTA is released and can be detected in supernatant fraction between different bacterial species as well as between strains of the same species. For some bacterial strains no signal might be detected.
11. The TEMED initiates the polymerization of the gel, hence TEMED should be added last and only added once the gel caster is ready.
12. Overlaying the gels with isopropanol creates a barrier to air at the top of the gel. Air inhibits the polymerization and would create an uneven line between the resolving and stacking gel, which would affect the quality of the gel electrophoresis and sharpness of the bands.

13. The combs are inserted already while the resolving gel is setting. The combs are removed after the resolving gel has set and reinserted again immediately after the addition of the stacking gel solution. Adding the combs already while the resolving gel is setting will hold the space at the top of the gel and make it easier to insert the comb into the stacking gel solution.
14. If the leftover gel mixture in the beaker has polymerized, the mixture has also polymerized in the gel casting system.
15. A small amount of 10% w/v bromophenol blue will give the stacking gel a light blue color, which will aid in locating the sample wells during the sample loading process.
16. The SDS-PAGE gels can either be used immediately after casting them or wrapped in a wet paper towel and then wrapped in cling film and stored at 4 °C for approximately 1 month. However, the bands will be crisper if the gels are used immediately or within the first few days of storage.
17. Typically, 10–15 μL cell extract sample is loaded per well on a 10-well gel and 3 μL of pre-stained protein marker. For a 15-well gel, usually 7.5–10 μL sample is loaded and 2.5 μL of pre-stained protein marker. However, the volume of sample that is loaded can be adjusted depending on the western blot signal strength obtained. Similarly, the volume of protein marker loaded can be adjusted depending on exact pre-stained protein marker used.
18. A Hoefer TE42 wet tank system requires approximately 5 L of transfer buffer and >10 gel can be processed at the same time; but smaller tank transfer systems can also be used. The transfer buffer can be topped up and reused for up to a month. Unused transfer buffer can be stored for several months at 4 °C.
19. In place of using 5% w/v dry milk as blocking solution, 3% w/v bovine serum albumin can be used.
20. The blocking step can also be performed overnight at 4 °C.
21. The antibody dilution and incubation time can be adjusted depending on LTA signal strength obtained. However, in general, we found that overnight incubation with the primary antibody at 4 °C gave a better signal, while the time and incubation temperature for the secondary antibody seemed less critical.
22. A plastic document wallet can be used for this step.
23. The side of a pipette tip can be used to distribute the ECL reagent across the entire surface of the membrane making sure not to scratch or tear the membrane.

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Chapter 9

Type I Lipoteichoic Acid (LTA) Purification by Hydrophobic Interaction Chromatography and Structural Analysis by 2D Nuclear Magnetic Resonance (NMR) Spectroscopy

Jeanine Rismondo and Angelika Gründling

Abstract

Type I lipoteichoic acid (LTA) is a glycerol phosphate polymer found in the cell envelope of diverse Gram-positive bacteria. The glycerol phosphate backbone is often further decorated with D-alanine and/or sugar residues. Here, we provide details of a 1-butanol extraction and purification method of type I LTA by hydrophobic interaction chromatography. The protocol has been adapted from methods originally described by Fischer et al. (Eur J Biochem 133:523–530, 1983) and further optimized by Morath et al. (J Exp Med 193:393–397, 2001). We also present information on a 2D nuclear magnetic resonance (NMR) analysis method to gain chemical and structural information of the purified LTA material.

Key words Type I LTA, 1-butanol LTA extraction, Hydrophobic interaction chromatography, FPLC purification of LTA, 2D NMR LTA analysis

1 Introduction

The bacterial cell envelope is a complex structure, composed of peptidoglycan and other cell wall polymers such as lipopolysaccharide (LPS) in Gram-negative bacteria and teichoic acids (TAs) in Gram-positive bacteria [1]. Teichoic acids in Gram-positive bacteria are further grouped into wall teichoic acid (WTA), which are covalently linked to the peptidoglycan layer and lipoteichoic acid (LTA) attached to the outer leaflet of the bacterial membrane by a lipid anchor [2–6]. LPS and TAs are critical for bacterial growth and play an important role in host-pathogen interactions, and hence have been extensively studied over the past decades (see reviews [2–6]). These studies have revealed not only many of the genetic determinants required for their synthesis but also highlighted the chemical diversity of the polymers between different bacterial species and strains. To aid the investigations on the structure and function of LPS and TAs, methods have been developed

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for their purification and chemical analysis. Here, we present a detailed protocol for the purification of type I LTA and its subsequent structural analysis by 2D nuclear magnetic resonance (NMR) spectroscopy. The method was adapted from procedures described by Fischer et al. [7] and subsequently optimized by Morath et al. [8]. By following the protocol described here, type I LTA of sufficient purity and quantities can be isolated for a subsequent structural 2D NMR analysis. Type I LTA is often used for immunological studies, where purity is of utmost importance. To obtain LTA of purity for immunological studies the original publications and some of the key follow-up studies should be consulted, which describe additional steps required to remove DNA and protein contaminations from samples and also highlight potential issues when using purified LTA material obtained from commercial sources [7–10]. To avoid any effects due to lipoprotein contaminations in LTA preparations, it might be advisable to isolate LTA for immunological studies not from wild-type bacteria but from mutant strains that are unable to produce lipoproteins [11, 12]. However, the impact of lipoprotein contamination in LTA preparation on downstream applications is debated [12, 13].

Type I LTA is a glycerol phosphate polymer usually linked by a glycolipid anchor to the outer leaflet of the cytoplasmic membrane in many Gram-positive bacteria belonging to the Bacillota phylum [5, 6]. Some of the glycerol phosphate backbone subunits are further decorated with D-alanine or sugar residues, such as galactose (Gal) residues in the case of *Listeria monocytogenes* (Fig. 1) or *N*-acetyl glucosamine (Glc*N*Ac) in the case of *Staphylococcus aureus* and *Bacillus subtilis* strains. The protocol for the purification of type I LTA is based on purification methods originally developed for the purification of LPS and was subsequently optimized to retain labile structural features on LTA such as D-alanine modifications, which are hydrolyzed at alkaline conditions or during the hot phenol extraction process originally used [7, 8]. Hence, the LTA purification is performed under low pH buffer conditions and now using a 1-butanol extraction method, which can be performed at room temperature [8]. The principal steps in the purification process are as follows: after the bacterial growth, the cells are collected and mechanically lysed for efficient LTA extraction [7, 8]. The lysed cells are then extracted using a 1-butanol/aqueous buffer mixture to separate the LTA from membrane lipids [8]. During this step the membrane lipids will partition into the organic 1-butanol phase, while the type I LTA will partition into the aqueous buffer phase due to its hydrophilic glycerol phosphate backbone structure. In our protocol, the extracted LTA is afterward purified by hydrophobic interaction chromatography over an octyl-Sepharose column. Additional purification steps to remove DNA and protein contaminations as well as additional or alternative purification steps using anion exchange chromatography have been described [7, 8]. After

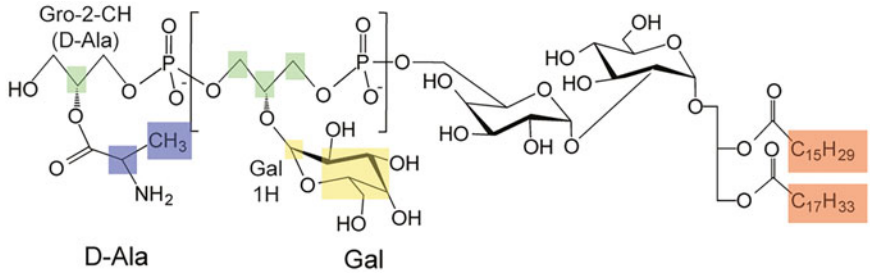

Fig. 1 Chemical structure of type I LTA from *L. monocytogenes*. Type I LTA in *L. monocytogenes* is a glycerol phosphate polymer of approximately 20 repeating units, which is linked by a glycolipid anchor to the bacterial membrane. The glycerol phosphate groups can be further modified with D-alanine or galactose residues (D-Ala or Gal). Locations of non-exchangeable protons contributing to peaks in NMR spectra shown in Fig. 3 are highlighted in different colors. Protons from the lipid anchor are highlighted in orange (58 per lipid anchor), non-exchangeable protons from the D-alanine substitutions (4 per D-Ala) are shown in blue, non-exchangeable protons from the glycerol phosphate subunits (5 per GroP subunit) in green and non-exchangeable protons from the galactose substitutions (7 per galactose) in yellow. The proton at the C1 position of the galactose residue yields a clearly separated peak on NMR spectra in the 5.2–5.3 ppm region and the proton at the C2 position of a glycerol phosphate subunit modified with a D-alanine in the 5.4 ppm area (*see* Fig. 3). (This figure is from Percy et al. [14], which was published under a Creative Commons CC BY 4.0 license (https:/creativecommons.org/licenses/by/4.0/))

the column chromatography, fractions containing the LTA polymer are identified by western blot using a polyglycerol-phosphate-specific commercially available antibody and using a method as described in detail in a separate protocol in this issue (*see* Millership and Gründling). The purified LTA material is subsequently dialyzed, concentrated, and prepared for 2D NMR analysis. Here, we describe the details for the purification and analysis of type I LTA extracted from *L. monocytogenes*. However, the protocol can be adapted for the purification and analysis of type I LTA from other bacterial species. As references, typical NMR spectra of type I LTA isolated from *L. monocytogenes, S. aureus,* and *B. subtilis* are also shown in this protocol.

2 Materials

All solutions should be prepared using ultrapure water (deionized water further purified to obtain a sensitivity of 18 MΩ-cm). The reagents are stored at room temperature unless otherwise specified. Some reagents will need to be handled using appropriate protective equipment and disposed of through dedicated waste routes and for this country and institution specific regulations and procedures should be followed.

2.1 Bacterial Growth

1. 500 mL and 2 L glass culture flasks (autoclaved).
2. Brain Heart Infusion (BHI) medium: Dissolve 37 g BHI powder per L water and sterilize by autoclaving (*see* **Notes 1** and **2**).
3. 1 M sodium citrate buffer, pH 4.7: Adjust pH with several milliliters of concentrated acetic acid.
4. 0.1 M sodium citrate buffer, pH 4.7: Mix 100 mL of 1 M sodium citrate buffer pH 4.7 with 900 mL water.
5. Shaking and static incubators set to 37 °C (*see* **Note 1**).
6. Sorvall centrifuge with rotor and centrifuge bottles to collect bacteria from a 2 L culture (e.g., Sorvall RC6+ centrifuge with F12-6X500LEX rotor).
7. Optional: −20 °C freezer.

2.2 Bacterial Lysis

1. 0.1 mm glass beads.
2. BeadBeater with a 100 mL homogenizing chamber (e.g., BioSpec) (*see* **Note 3**).
3. 50 mL centrifuge tubes suitable for centrifugation speeds of 12,500 × *g* (*see* **Note 4**).
4. 0.1 M sodium citrate buffer, pH 4.7 (*see* Subheadings 2.1, **items 3** and **4**).
5. Sorvall centrifuge with rotor for spinning 50 mL centrifuge tubes (e.g., Sorvall RC6+ centrifuge with F13-14x50cy rotor) (*see* **Note 4**).
6. Scale.
7. Cold room.
8. Optional: −20 °C freezer.

2.3 1-Butanol Extraction

1. 1-Butanol.
2. 0.1 M sodium citrate buffer, pH 4.7 (*see* Subheadings 2.1, **items 3** and **4**).
3. 20 mM sodium citrate buffer, pH 4.7: Mix 980 mL water with 20 mL 1 M sodium citrate pH 4.7 buffer (*see* Subheading 2.1, **item 3** for preparing the 1 M stock solution).
4. Sorvall centrifuge with rotor for spinning 50 mL centrifuge tubes (e.g., Sorvall RC6+ centrifuge with F13-14x50cy rotor) (*see* **Note 4**).
5. 25 mL glass pipettes.
6. 250 mL glass beaker.
7. Magnetic stirrer and stir bar.
8. Glass Pasteur pipettes.
9. Aluminum foil.

10. 2–5 L plastic beakers.
11. 1-kDa cut-off dialysis membrane (*see* **Note 5**).
12. Scale.
13. Cold room.
14. Chemical Hood.
15. Optional: −20 °C freezer.
16. Optional: Clips for dialysis membranes.

2.4 Column Purification (see Notes 6–8)

1. 1-Propanol.
2. 1 M sodium citrate buffer, pH 4.7 (*see* Subheading 2.1, **item 3** and **Note 9**).
3. 0.2 μM pore size nylon membrane filters.
4. Reusable bottle top filtration device.
5. Equilibration buffer: 0.1 M sodium citrate buffer, pH 4.7, with 15% 1-Propanol. Mix 750 mL water, 150 mL 1-Propanol, 100 mL 1 M sodium citrate buffer, pH 4.7 and filter solution through a 0.2 μM pore size nylon membrane filter (*see* **Notes 9** and **10**).
6. Buffer A: 50 mM sodium citrate buffer, pH 4.7, with 15% 1-Propanol. Mix 800 mL water, 150 mL 1-Propanol, 50 mL 1 M sodium citrate buffer, pH 4.7 and filter solution through a 0.2 μM pore size nylon membrane filter (*see* **Notes 9** and **10**).
7. Buffer B: 50 mM sodium citrate buffer, pH 4.7, with 60% 1-Propanol. Mix 350 mL water, 600 mL 1-Propanol, 50 mL 1 M sodium citrate buffer, pH 4.7 and filter solution through a 0.2 μM pore size nylon membrane filter (*see* **Notes 9** and **10**).
8. H_2O filtered through a 0.2 μM pore size membrane filter.
9. 20% Ethanol filtered through a 0.2 μM pore size membrane filter.
10. 1 M NaOH filtered through a 0.2 μM pore size nylon membrane filter.
11. 50 mL centrifuge tubes (*see* **Note 4**).
12. Sorvall centrifuge with rotor for spinning 50 mL centrifuge tubes (e.g., Sorvall RC6+ centrifuge with F13-14x50cy rotor).
13. FPLC machine and fraction collector for 60 or more tubes (e.g., Äkta purifier with Frac 950 fraction collector).
14. 24 cm × 1.6 cm diameter octyl-Sepharose column.
15. 50 mL chromatography sample loading Superloop.
16. Sample collection tubes for FPLC machine.

17. SDS-PAGE sample buffer (1×): 0.0625 M Tris pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue, 10% β-mercaptoethanol.
18. pH paper.

2.5 LTA Detection

Reagents needed and a detailed protocol for the detection of type I LTA by western blot is presented in a different protocol in this book (please *see* Millership and Gründling).

2.6 Sample Dialysis

1. 5 L plastic beakers with water.
2. 250 mL glass beaker.
3. 1-kDa cut-off dialysis membrane.
4. Cold room.

2.7 Freeze Drying

1. −80 °C Freezer.
2. Liquid nitrogen.
3. Freeze dryer with pump for drying >120 mL volumes (e.g., CRIST Alpha 1–2 LDplus freezer dryer, with vacuubrand RZ 2.5 pump).

2.8 NMR Sample Preparation

1. 99.96% D_2O (1.5 mL/per sample).
2. 2 mL screw cap tubes.
3. Fine scale (accuracy of ± 0.1 mg).
4. Needle.
5. Liquid nitrogen.
6. Freeze dryer (*see* Subheading 2.7, **item 3**).

2.9 NMR Analysis

1. NMR tubes: 5 mm, thin-walled, borosilicate glass ASTM E438, Type I, class B.
2. 600-MHz Bruker Advance III NMR spectrometer equipped with a TCl cryoprobe.

3 Methods

3.1 Bacterial Growth for LTA Extraction

1. In the morning, inoculate 50 mL BHI medium in a 500 mL culture flask with a single colony of the required *L. monocytogenes* strain and incubate the culture at 37 °C with shaking at 180 rpm (*see* **Notes 1** and **2**).
2. In the evening, inoculate 2 × 1 L culture flasks each containing 1 L BHI medium with 10 mL of the over-day culture (*see* **Notes 1** and **2**).
3. Incubate the cultures overnight at 37 °C with 180 rpm shaking (*see* **Notes 1** and **2**).

4. Next day, cool the cultures for 1 h on ice.
5. Transfer the culture to centrifuge bottles and collect the bacteria by centrifugation for 10 min at 6100 × *g* and 4 °C.
6. Discard the supernatant and suspend the bacterial pellet in a final volume of 45 mL cold 0.1 M sodium citrate buffer, pH 4.7, keeping the cells on ice.
7. Pellet the bacteria again by centrifugation for 10 min at 6100 × *g* and 4 °C.
8. Discard the supernatant and freeze the bacterial pellet at −20 °C (or continue with the next steps).

3.2 Bacterial Lysis

1. Suspend the bacterial pellet in 40 mL cold 0.1 M sodium citrate buffer, pH 4.7.
2. Add 40 mL of 0.1 mm glass beads to BeadBeater homogenizing chamber and add the bacterial cell suspension to the beads in the chamber.
3. Close the homogenizing chamber and mix the cells and glass beads by inverting the chamber several times and subsequently fill the homogenizing chamber completely with cold 0.1 M sodium citrate buffer, pH 4.7.
4. Lyse the cells by performing 5 × 2 min BeadBeater cycles. Between each cycle, put the sample on ice for 2 min. Before each cycle, shake the chamber to mix cells and glass beads. This step is preferably performed in a cold room (*see* **Note 3**).
5. After the final bead beating step, shake the chamber to mix the cells and glass beads and transfer the sample into 2 × 50 mL centrifuge tubes per sample.
6. Spin the tubes for 1 min at 200 × *g* and 4 °C to settle the glass beads.
7. Transfer the supernatant to two new 50 mL centrifuge tubes.
8. Add 20 mL cold 0.1 M sodium citrate buffer, pH 4.7, to each tube containing the glass beads and vortex briefly.
9. Spin the tubes again for 1 min at 200 × *g* and 4 °C to settle the glass beads.
10. Combine the supernatant containing the lysed cells with the supernatant from **step 7**.
11. Centrifuge the tubes with the lysed cells again for 1 min at 200 × *g* and 4 °C to remove any residual glass beads.
12. Transfer the supernatant to two new 50 mL centrifuge tube and fill each tube up to 45 mL with cold 0.1 M sodium citrate buffer, pH 4.7.
13. Collect the lysed cells by centrifugation for 45 min at 12,500 × *g* and 4 °C (*see* **Note 4**).

14. Remove the supernatant and combine and suspend the pellets of the lysed cells from the two tubes in 45 mL final volume of cold 0.1 M sodium citrate buffer, pH 4.7.
15. Spin the samples again for 45 min at 12,500 × *g* and 4 °C to collect the lysed cells and remove the supernatant (*see* **Note 4**).
16. Determine the wet weight of the bacterial cell pellet and freeze the pellet at −20 °C or continue with the next step.

3.3 1-Butanol Extraction

1. Fill 2× 2–5 L plastic beakers with 2 L of 20 mM sodium citrate buffer, pH 4.7 each for dialysis. Add a stir bar to each beaker. Cover the beaker with aluminum foil and precool to 4 °C.
2. Suspend the pellet with the lysed cells from Subheading 3.2, **step 16** in cold 0.1 M sodium citrate buffer, pH 4.7, to a final wet weight of 0.4 g per mL buffer.
3. Transfer the sample into a 250 mL glass beaker.
4. Under the chemical hood, add an equal volume of 1-Butanol using a 25 mL glass pipette. Add a clean stir bar.
5. Cover the beaker with aluminum foil and stir vigorously at room temperature for 30 min.
6. Under the fume hood, pour the suspension into a 50 mL centrifuge tube and centrifuge the samples for 25 min at 12,500 × *g* and 4 °C (*see* **Note 4**).
7. After the centrifugation, there will be two phases and a cell pellet at the bottom. Under the fume hood, carefully transfer the upper and lower phase by pouring the liquid into a new 50 mL centrifuge tube. Use a glass Pasteur pipette to transfer the remaining liquid. Avoid transferring any of the cell pellet from the bottom of the tube.
8. Spin the sample for 25 min at 12,500 × *g* and 4 °C (*see* **Note 4**).
9. Under the fume hood and using a glass Pasteur pipette, transfer the bottom, aqueous phase into a new 50 mL centrifuge tube (*see* **Notes 11** and **12**).
10. Cut a 1-kDa cut-off dialysis membrane to an appropriate length for dialysis. The volume of the sample will only slightly increase. Rinse the membranes 3× with 50 mL water and 1× with 50 mL 20 mM sodium citrate buffer, pH 4.7 (*see* **Note 5**).
11. Transfer the sample into the 1-kDa cut-off dialysis membranes. To do this, tie a double knot at one end of the dialysis membrane and transfer the sample into the dialysis tubing using a pipette. Remove all air bubbles and make a double knot on the other end of the dialysis membrane.
12. Dialyze the sample against 2 L of cold 20 mM sodium citrate, pH 4.7 (*see* Subheading 3.3, **step 1**) buffer for 2 h at 4 °C with gentle stirring.

13. After 2 h, place the sample into the second beaker containing 2 L of 20 mM sodium citrate, pH 4.7 and dialyze overnight with gentle stirring.
14. After the dialysis, cut the dialysis tubing open and transfer the sample to a 50 mL centrifuge tube. Store the sample at −20 °C or proceed to Subheading 3.4, **step 4**.

3.4 Sepharose Column Purification of LTA

1. Perform a pump wash for pump A with inlet tube in equilibration buffer and for pump B with inlet tube at this point still in water.
2. Equilibrate the 24 cm × 1.6 cm octyl Sepharose column with 100 mL of equilibration buffer at a flow rate of 0.5 mL per min. Set the pressure alarm to 0.5 Mbar and the timer to end after 200 min (*see* **Note 13**).
3. Turn on the UV light of the FPLC machine at least 15 min before loading the sample.
4. Prepare the LTA sample obtained in Subheading 3.3, **step 14** for the FPLC step by adjusting the sample to give a final concentration of 15% 1-Propanol and 0.1 M sodium citrate, pH 4.7: e.g., to 30 mL dialyzed LTA sample, add 3.4 mL 1 M sodium citrate buffer, pH 4.7, 6 mL 1-Propanol and 0.6 mL water, to yield a final volume of 40 mL.
5. Spin the sample at 6100 × *g* for 10 min at 4 °C to remove any debris and transfer the sample to a new 50 mL centrifuge tube.
6. Take a 20 μL aliquot of the sample and mix with 40 μL of 1× SDS sample buffer and store at −20 °C for the subsequent western-blot analysis (see steps described under Subheading 3.5).
7. Load the LTA sample into a Superloop and connect the loop to the FPLC machine as per the manufacturer's instructions.
8. Load the sample onto the equilibrated octyl-Sepharose column: Pump A must be in equilibration buffer. Use a flow rate of 0.5 mL per min to inject the sample. Set the pressure alarm to 0.5 Mbar. Calculate the end time in accordance with sample volume (i.e., for a 32 mL sample, use 30 mL of the sample at a flow rate of 0.5 mL/min and set the end timer to 60 min) (*see* **Note 13**).
9. After loading the sample, wash the column with 120 mL of equilibration buffer at a flow rate of 0.5 mL per min. Set the pressure alarm to 0.5 Mbar and the timer to end after 240 min (*see* **Note 13**).
10. Place pump A inlet into buffer A and perform a pump wash and pump B inlet tube into buffer B and perform a pump wash.

11. Fill the fraction collector with 60 tubes.
12. Elute the LTA from the column using a linear gradient of buffer B from 0% to 100% buffer B over 200 mL at a flow rate of 0.5 mL/min, followed by 100 mL 100% buffer B at a flow rate of 0.5 mL/min. Collect 5 mL elution fractions. Set the pressure alarm to 0.5 Mbar (*see* **Note 13**).
13. Turn the UV light of the machine off until again needed.
14. If a second LTA sample is purified, start the FPLC purification protocol again from Subheading 3.4, **step 1**.
15. For cleaning the column after 2 to 4 runs or for storing the column after use, place pump A and pump B inlets into water and perform a pump wash for each pump. Repeat the pump wash for pump B a second time.
16. Wash the column with 60 mL water at a flow rate of 0.5 mL/min. Set the pressure alarm to 0.5 Mbar and the timer to end after 120 min (*see* **Note 13**).
17. Put the pump A inlet tube into 1 M NaOH and perform a pump wash for pump A.
18. Wash the column with 120 mL 1 M NaOH at a flow rate of 0.5 mL/min. Set the pressure alarm to 0.5 Mbar and the timer to end after 240 min (*see* **Note 13**).
19. Put the pump A inlet tube into water and perform a pump wash for pump A.
20. Wash the column with at least 150 mL water at a flow rate of 0.5 mL/min. Set the pressure alarm to 0.5 Mbar and the timer to end after 300 min. Wash until the pH of the water at the outlet is again neutral. Use pH paper to check the pH of the liquid at the outlet (*see* **Note 13**).
21. Put the pump A inlet tube into equilibration buffer and perform a pump wash for pump A.
22. Wash the column with 100 mL equilibration buffer at a flow rate of 0.5 mL/min. Set the pressure alarm to 0.5 Mbar and the timer to end after 200 min. Now the column is ready to be used again or stored (*see* **Note 13**).
23. Put the pump A and pump B inlet tube into H_2O and perform a pump wash for pump A and pump B.
24. Put the pump A and pump B inlet tube into 20% Ethanol and perform a pump wash for pump A and pump B.

3.5 Detection of LTA by Western Blot (Fig. 2)

1. Take 20 µL of each FPLC fraction starting from fraction 18 up to fraction 44 and mix each fraction with 60 µL of 1× SDS sample buffer. The LTA is usually detected in fractions 20–35.

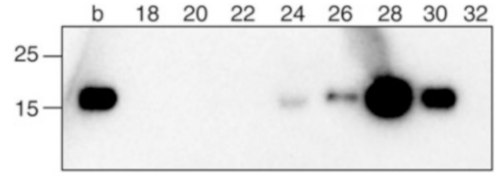

Fig. 2 Detection of LTA in FPLC fractions by western blot. 10 μL of every second FPLC fraction (from fractions 18–32) were separated on a 15% SDS polyacrylamide gel, transferred to a PVDF membrane and the LTA detected by western-blot using a polyglycerolphosphate-specific antibody. A sample taken before the column purification (labelled as **b**) was used as a positive control

2. Boil the sample aliquots for 20 min. The sample that was taken after the dialysis step (*see* Subheading 3.4, **step 6**) also needs to be boiled at this point.
3. Spin all samples for 5 min at 17,000 × *g* at room temperature.
4. Load 10 μL onto a 15% SDS polyacrylamide gel alongside a pre-stained protein ladder.
5. Continue with the LTA western blot as described in a different protocol in this book (please *see* Millership and Gründling).

3.6 LTA Dialysis

1. Fill two 5 L plastic beakers with 4 L of water each day before use, cover them with aluminum foil and precool to 4 °C.
2. Combine fractions that gave a signal on the LTA western blot in a 50 mL centrifuge tube.
3. Cut a 1-kDa cut-off dialysis membrane and wash it three times with 50 mL water. The sample volume will increase up to 3–4 times, so a larger piece of dialysis membrane should be prepared.
4. Transfer the sample into the 1-kDa cut-off dialysis membrane cut to approximately twice the length as needed for the starting volume. Tie a double knot at one end of the membrane and transfer the sample into the dialysis tubing using a pipette. Remove all air bubbles, leaving some unfilled dialysis tubing, and tie a double knot on the other end of tubing.
5. Dialyze the sample against water at 4 °C with gentle stirring (*see* **Note 14**).
6. Change the dialysis twice a day for 5–7 days.
7. After the final dialysis step, cut the dialysis tubing open and transfer the sample to a clean 250 mL glass beaker and proceed immediately to next step.

3.7 Freeze Drying of the LTA Sample

1. For freeze drying the LTA sample obtained at Subheading 3.6, **step 7**, transfer the sample into multiple 50 mL disposable centrifuge tubes, filling each tube with 15–20 mL sample. Here, a freeze-drying method using 50 mL disposable

centrifuge tubes and a CHRIST Alpha 1–2 LDplus freezer dryer with a vacuubrand RZ 2.5 pump is described. But the freeze-drying step can be adjusted depending on the equipment available in the laboratory.

2. Use a needle make 2–3 holes into the lid of each centrifuge tube.
3. Freeze the sample in a −80 °C freezer. Rotate the tubes throughout the freezing process (approx. every 5–10 min).
4. Turn on the freeze dryer ahead of time to let it cool down.
5. Place the samples into liquid nitrogen for 5 min and subsequently add the samples to the freezer dryer and freeze dry the sample until completely dry (*see* **Note 15**).
6. Suspend the dried LTA sample in 1 mL water (*see* **Note 16**).
7. Weigh a 2 mL screw cap tube three times on a fine balance and calculate the average weight of the tube.
8. Transfer the LTA sample into the weighed 2 mL screw cap tube.
9. Use a needle make a hole into the lid of the tube.
10. Freeze the samples for 5 min in liquid nitrogen.
11. Turn on the freeze dryer ahead of time to let it cool down.
12. Put the LTA samples into the freeze dryer and freeze dry overnight or until the sample is completely dry.
13. Remove the tube with the dried LTA sample from the freeze dryer.
14. Measure the weight three times on a fine balance. Take the average weight and calculate the amount of LTA in mg.
15. Proceed to the next step or store the LTA powder at −20 °C until further use. For storage, the lid of the tube should be changed with a new lid without holes.

3.8 Sample Preparation for NMR Analysis

1. Freeze dry 2 mg of LTA overnight. For this, suspend the LTA sample obtained in Subheading 3.7, **step 15** in 1 mL water and transfer the amount that corresponds to 2 mg LTA into a new 2 mL screw cap tube and freeze dry the sample as described in Subheading 3.7, **steps 9–13**.
2. Suspend the dried LTA sample in 500 µL 99.96% D_2O and freeze dry the sample again as described in Subheading 3.7, **steps 9–13**.
3. Suspend the dried LTA sample again in 500 µL 99.96% D_2O and freeze dry the sample again as described in Subheading 3.7, **steps 9–13**.
4. Right before the NMR experiment, suspend the dried LTA sample in 500 µL 99.96% D_2O.

3.9 NMR Analysis of LTA

1. Transfer 200 μL of the LTA sample into a 5 mm NMR tube.
2. Insert the NMR tube into the NMR machine. We usually perform the NMR analysis on a 600-MHz Bruker Advance III spectrometer equipped with a TCl cryoprobe.
3. Record the NMR spectrum at 303 K with a total recycling time of 5 s and a ^{1}H flip angle of around 30°.
4. To determine the chain length of the LTA and the percentage of D-alanine and sugar modifications, the different LTA-specific peaks are integrated as described in Percy et al. [14] and as shown in Fig. 3. In this figure, the peaks derived from the non-exchangeable protons from the fatty acids are highlighted in orange, from D-alanines in blue, from glycerol phosphate subunits in green, and from the sugar modifications (if present) in yellow. If sugar modifications are present, the signal in the 4-ppm region (highlighted in green and yellow in Fig. 3) is derived from both, sugar and glycerol phosphate protons. If galactose substitutions are present, the signal from one of the sugar protons is observed in the 5.2–5.3 ppm region (Figs. 1 and 3a, b). If Glc*N*Ac modifications are present, the signal from one of the sugar protons is also observed in the 5.2–5.3 ppm and additional proton signatures are observed in the 2 and 3.5 ppm regions (Fig. 3c) (*see* **Notes 17** and **18**).
5. For the quantification, set the area for the peak at 4.3 ppm (CH group of D-alanine) to 1 or the area for the peak at 1.6 ppm (CH_3 group of D-alanine) to 3 (highlighted in blue in Fig. 3).
6. Based on the integration values of the other peaks and the number of non-exchangeable protons determine the LTA chain length and % D-alanine and % sugar modification. The following numbers of non-exchangeable protons should be used: 58 protons for the lipid anchor in *L. monocytogenes*, 5 protons for each glycerol phosphate subunit (GroP), 4 protons for each D-alanine substitution, and 7 protons total for each galactose modifications (*see* **Note 18**).
7. To calculate the percentage of substitution, divide the proton-adjusted value for D-alanine or the proton-adjusted value of galactose by the proton-adjusted value for GroP and multiply by 100.
8. To calculate the chain length, divide the proton-adjusted value of the GroP signal by the proton adjusted-value of the fatty acid signal.

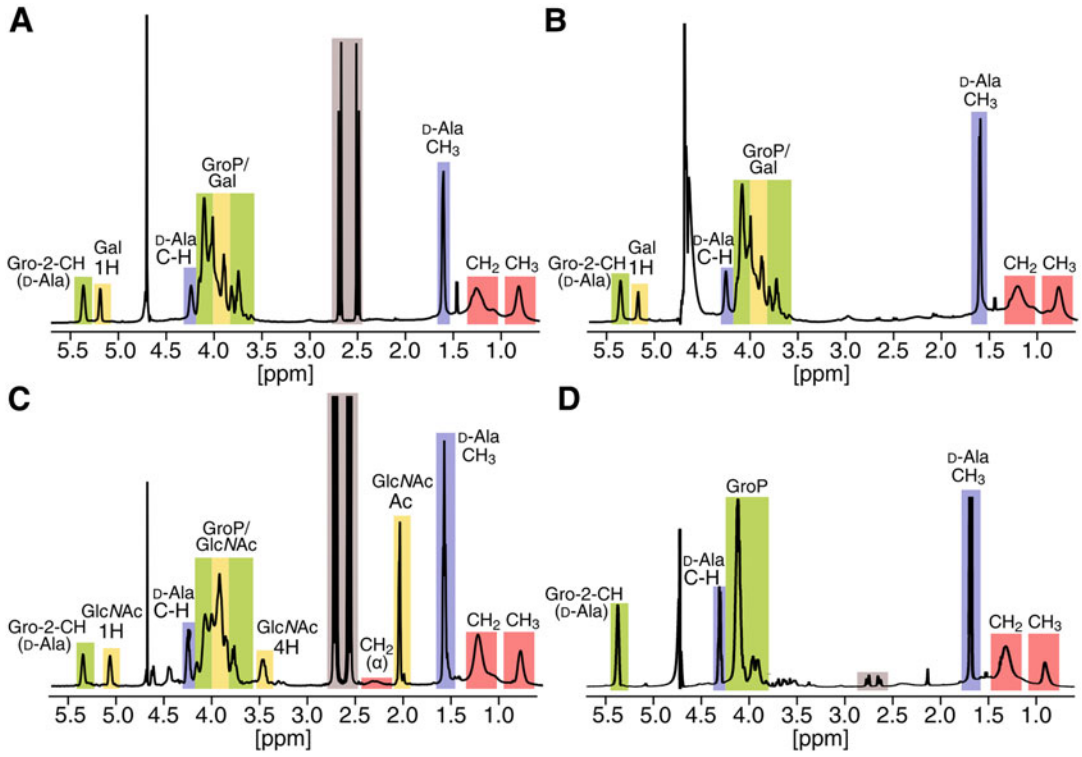

Fig. 3 NMR spectra of LTA isolated from *L. monocytogenes*, *B. subtilis* and *S. aureus*. (**a**, **b**) NMR spectra of LTA isolated from *L. monocytogenes* strain 10403S using the full protocol (**a**) or the quick protocol (**b**). (**c**, **d**) NMR spectra of LTA isolated from *B. subtilis* 168 (**c**) and *S. aureus* RN4220 (**d**). Colored boxes and labels indicate the non-exchangeable protons derived from the different LTA components (orange: lipid anchor; blue: D-alanine, green: glycerol phosphate, yellow: sugar modifications). Peaks were assigned in accordance with previous studies [8, 9, 14, 16] and it is also of note that the signal in the 4-ppm region is derived from both glycerol phosphate and sugar protons if present (highlighted in both green and yellow). Residual citrate, a buffer component used during the LTA purification process, is marked by gray boxes. Gal galactose, Glc*N*Ac *N*-acetyl glucosamine, D-Ala D-alanine, GroP glycerol phosphate. The chemical structure of type I LTA from *L. monocytogenes* is shown in Fig. 1

3.10 Quick Protocol for LTA Analysis Isolated from L. monocytogenes *(see Note 19)*

1. Grow and lyse the *L. monocytogenes* bacteria as described in Subheadings 3.1 and 3.2.
2. Perform the 1-butanol extraction as described in Subheading 3.3 until and including **step 9**, except for Subheading 3.3, **step 1**, prepare 2× 5 L plastic beakers with 4 L water each in place of the 20 mM sodium citrate buffer, pH 4.7.
3. Following Subheading 3.3, **step 9**, spin the sample for 10 min at 6100 × *g* to remove any remaining debris.
4. Cut a 1-kDa cut-off dialysis membrane and wash it three times with 50 mL water. The sample volume will increase slightly during the dialysis step.

5. Transfer the sample into the 1-kDa cut-off membrane: Tie a double knot at one end of the membrane and transfer the sample into the dialysis tubing using a pipette. Remove all air bubbles and tie a double knot on the other end of the membrane.
6. Dialyze the sample against water at 4 °C with gentle stirring (*see* **Note 14**).
7. Change the dialysis twice a day for 2–3 days.
8. Continue with the freeze-drying process, sample preparation for NMR analysis, and NMR analysis as described in Subheadings 3.7–3.9.

4 Notes

1. The growth medium will depend on the bacterial strains analyzed. We have performed the LTA extraction and NMR analysis method using *Staphylococcus aureus* cultures grown in Tryptic Soy broth (TSB), *Bacillus subtilis* cultures grown in Lysogenic Broth (LB) supplemented with 0.1% glucose and *Listeria monocytogenes* cultures grown in BHI medium. *B. subtilis* 168 was grown at 30 °C, all other strains were grown at 37 °C. LTA samples can either be prepared from overnight cultures or from log-phage cultures.
2. For *S. aureus*, we have performed LTA extractions from 6 L TSB medium and log-phase cultures. For *B. subtilis*, we used a 2 L overnight culture for the LTA purification protocol.
3. The rubber of the homogenizing chambers can disintegrate over time. Hence, the chambers should be checked before use, to ensure they do not leak. Sometimes, the sample will turn slightly grey instead of remaining white due to small rubber particles getting into the sample; however, this does not seem to impact the LTA purification and subsequent NMR analysis.
4. At several steps in this protocol, 50 mL tubes which can be centrifuged at 12,500 × *g* are needed. Hence the tube specifications need to be checked before use and the appropriate high-speed centrifuge rotor needs to be available in the laboratory. Alternatively, multiple SS34 tubes can be used together with, e.g., a Sorvall SS-34 fixed-angle rotor.
5. The membrane is stored in water containing azide, hence gloves should be worn while handling the membrane and the membrane should be rinsed before use.
6. All buffers should be filtered and pre-cooled to 4 °C before use.

7. We use glassware that is dedicated for the LTA extractions including glass bottles and measuring cylinders, which are free of detergent.
8. In this protocol, the LTA is purified by hydrophobic interaction chromatography using an octyl-Sepharose column. In other protocols, an additional purification step or alternative purification step is performed using an anion exchange column [7, 8].
9. In the original LTA purification protocol by Morath et al. [8], ammonium acetate buffers and not sodium citrate buffers are used for column purification.
10. Add the sodium citrate buffer last for all buffers and filter all solutions through a 0.2 μM pore size nylon membrane filter before use on the FPLC machine.
11. For LTA extractions from *S. aureus* strains, it is recommended to repeat the 1-butanol extraction a second time. To do this, mix the aqueous phase obtained after Subheading 3.3, **step 9** again with an equal volume of 1-butanol and stir vigorously at room temperature for 30 min. Under the fume hood, poor the sample into a new 50 mL centrifuge tube and spin for 25 min at 12,500 × *g* at 4 °C. Collect the bottom aqueous phase and continue with the dialysis step (Subheading 3.3, **step 10**).
12. In the original purification methods, the extracted sample is at this point further treated with DNase and in same protocols also with protease [7, 8], however we found that by following the steps described in this protocol, LTA of sufficient purity is obtained for subsequent 2D NMR analysis.
13. If the pressure exceeds 0.5 Mbar, reduce the flow rate to avoid damage to the column.
14. Multiple samples can be dialyzed in the same beaker. If doing this, the samples need to be labeled appropriately (e.g., by adding clips with a different color to the end of the tubing).
15. Using the described setup for freeze drying it will take around 48–60 h for the samples to be completely dry.
16. The color of the powder differs between organisms. The powder is yellow for *L. monocytogenes* and white for *B. subtilis*.
17. We usually use the TopSpin software (Bruker Biospin, Ltd.) for the peak integration step and MNova program (Mestrelab Research, S.L.) for better visualization of the NMR spectra (e.g., for the preparation of figures).
18. The number of non-exchangeable protons for the LTA isolated from *B. subtilis* is 58 protons for the lipid anchor, 5 for GroP, 4 for D-alanine, and 10 for *N*-acetyl glucosamine (Glc*N*Ac) [15]. The number of nonexchangeable protons for the LTA

isolated from *S. aureus* is 59 protons for the lipid anchor, 5 for GroP, 4 for D-alanine, and 10 for Glc*N*Ac, if present.

19. For the NMR analysis of type I LTA from *L. monocytogenes,* a shorted version of the protocol described here in which the FPLC purification step is omitted can be used. We also tried to perform the quick LTA purification and NMR analysis protocol with *B. subtilis* samples, but the purity of the sample and the resolution of the NMR peaks were insufficient to adequately determine the LTA structure.

Acknowledgments

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Chapter 10

Single-Copy Gene Editing of a Cell Wall-Anchored Pilin in *Actinomyces oris*

Aadil H. Bhat, Asis Das, and Hung Ton-That

Abstract

The Gram-positive bacterium *Actinomyces oris* expresses a unique cell wall-anchored fimbria comprised of the fimbrial shaft FimA and the tip fimbrillin CafA, whose gene is not genetically linked to the *fimA* locus, unlike many other fimbrial gene loci in Gram-positive bacteria. Mutational analyses of individual fimbrillins, FimA and CafA, in *A. oris* often rely on multi-copy plasmids that may alter the stoichiometry of fimbrillins in vivo, hence fimbrial assembly. Here, we provide a robust method for single-copy gene expression and mutagenesis in *A. oris*, using CafA as an experimental model. This method can be applied for single-copy gene editing in various bacterial systems.

Key words *Actinomyces oris*, Homologous recombination, Co-integration, Pilin, Room temperature transformation, Mutagenesis

1 Introduction

Actinomyces oris is a Gram-positive oral actinobacterium that plays a key role in the development of oral biofilms, or dental plaque, because of its ability to interact with many oral microbes via heterodimeric fimbriae, or pili. *A. oris* produces two types of cell wall-anchored fimbriae, type 1 and type 2, with the latter essential for polymicrobial interaction, or coaggregation, and biofilm formation [1, 2]. Initially, it was found that type 2 fimbriae are made of the fimbrial shaft FimA and tip fimbrillin FimB [3], both of which harbor a cell wall sorting signal (CWSS) comprised of an LPXTG motif, followed by a hydrophobic domain and a positively charged tail [4]. Assembly of type 2 fimbriae on the bacterial cell wall requires two sortase enzymes, SrtC2 and SrtA; the former catalyzes fimbrial polymerization, linking individual fimbrillins into covalently linked polymers with FimB at tip [3], while the latter anchors the resulting fimbrial polymers to peptidoglycan [5]. Interestingly, a CWSS-containing protein, named CafA, whose gene is not within

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the *fimB-fimA-srtC2* locus, hijacks the sortase machine SrtC2, forming a distinct fimbrial tip with the shaft FimA [2]. Unlike FimB, which is dispensable for bacterial coaggregation [1], CafA is essential for this process as deletion of *cafA* abrogates coaggregation [2]. Both tip fimbrillins are required for fimbrial assembly since a mutant strain lacking *fimB* and *cafA* is unable to assemble type 2 fimbriae [2].

The stoichiometry of fimbrillins and sortase enzymes appears to be critical for optimal fimbrial assembly, since deletion of the housekeeping sortase gene *srtA* leads to extensive elongation of fimbriae that fails to mediate coaggregation, while inducible expression of *srtA* alters fimbrial length [6]. Consistent with the notion of substrate-enzyme stoichiometry, overexpression of CafA from a multi-copy plasmid also appears to affect fimbrial assembly [2].

In *A. oris*, pJRD215 is the only multi-copy shuttle plasmid frequently used for complementation and mutagenesis [7]. This can present a challenge if overexpression of genes from this vector causes adverse phenotypes as in the case of SrtA and CafA. Here, we describe a protocol for single-copy gene editing, via endogenous homologous recombination, using *A. oris cafA* as an experimental model. In principle, this procedure can be used for site-directed mutagenesis, replacement and deletion of genes. Other advantages over episomal (replicative) plasmid-based expression include enhanced stability, reduced cell-to-cell variation, and robust mutagenesis for large genes, making it a better alternative [8].

2 Materials

2.1 Mutagenesis, PCR Amplification, and Overlapping PCR

1. Genomic DNA from *A. oris* MG1 [9].
2. Phusion High Fidelity DNA Polymerase (NEB).
3. 5X Phusion GC buffer (NEB).
4. 10 mM dNTPs.
5. DMSO.
6. Nuclease-free water.
7. Primers:

   F1 (forward) primer: 5'-GCGGCCGCGGCCGCG GGGAGCGCGGCCGACACC.

   R1 (Reverse) primer: 5'-AACTCATGAGTCCAGGGCCCGC .

   F2 primer: 5'-GTACGTCTAGATGTCCCTGGCCAAGC AGGTG.

   R2 primer: 5'-CGCGGCCGCGGCCGCGGCGGGCACC GCCGACTTG.

   R3 primer: 5′- GAGTGAGCTGATACCGCTCG.

8. 0.1 mL PCR tubes.
9. Thermocycler.
10. NanoDrop 1000 spectrophotometer.
11. QIAquick gel extraction kit.

2.2 Co-Integration Plasmid Construction

1. Chemically competent *E. coli* DH5α.
2. LB broth.
3. LB agar plates containing 50 μg/mL kanamycin.
4. pHTTh plasmid, derived from the vector pHTT177 [10], harbors an HpaI restriction enzyme site.
5. Apex 2X RED Taq Master Mix.
6. XbaI and HpaI restriction enzymes.
7. 10X CutSmart buffer.
8. T4 DNA ligase.
9. 50 μg/mL kanamycin.
10. Nuclease-free water.
11. Sterile 1.5 mL, 15 mL, and 50 mL centrifuge tubes.
12. Sterile spreaders and metal loops.
13. 37 °C incubators.
14. Water bath at 42 °C.
15. Plasmid miniprep kit.
16. NanoDrop 1000 spectrophotometer.

2.3 A. oris Competent Cell Preparation and Electro-Transformation

1. *Actinomyces oris* MG1 [9].
2. Heart infusion broth (HIB).
3. Heart infusion agar (HIA) plates.
4. HIA plates containing 40 μg/mL kanamycin.
5. 15% glycine in HIB, filter sterilized.
6. 50 μg/mL kanamycin.
7. Nuclease-free water.
8. 2-mm gap electroporation cuvettes.
9. Electroporator.
10. Sterile spreaders and metal loops.
11. 30 °C and 37 °C-shaker incubators with 5% CO_2.
12. 30 °C and 37 °C water bath shakers.
13. Disposable cuvettes.
14. Spectrophotometer.

2.4 Integrant Selection

1. HIA plates containing 40 μg/mL kanamycin.
2. Sterile toothpicks.
3. 200 μL sterile tips.
4. Phusion High-Fidelity DNA Polymerase.
5. 10 mM dNTPs.
6. DMSO.
7. QIAquick gel extraction kit.
8. 0.1-mL PCR tubes.
9. Thermocycler.

3 Methods

In this protocol, we provide an example of generating a CafA mutant, in which the LPXTG motif of the C-terminal CWSS is mutated to all alanine residues (*see* Fig. 1). In principle, any parts of CafA can be mutated or replaced.

3.1 Mutagenesis and PCR Amplification

1. Using the OligoAnalyzer tool and NEB Tm calculator, design forward (F1) and reverse (R1) primers to amplify the 3′ region of *cafA* gene coding for the CWSS (*see* Fig. 1). Primer F1 contains a 15-bp overhang with intended mutated bases at the 5′-end (*see* **Note 1**).

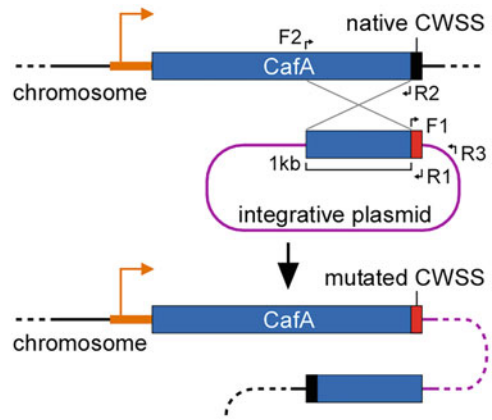

Fig. 1 Single-copy gene editing of CafA. An integrative plasmid (pHTTh), harboring a 1-kb segment homologous to the sequence upstream of the native CWSS (black) of CafA and a segment coding for a mutated CWSS (red), is constructed by overlapping PCR, using two primer sets, F2/R2 and F1/R1 respectively. Electroporation of this plasmid into *A. oris* MG1 permits homologous recombination, leading to insertion of the plasmid to the bacterial chromosome and generating a CafA mutant allele and a promoter-less *cafA* fragment

2. Prepare a PCR reaction mixture containing 10 μL of 5X Phusion GC Buffer, 1 μL of 10 mM dNTPs, 1.5 μL of 10 μM forward primer, 1.5 μL of 10 μM reverse primer, ~100 ng of template DNA, 2.5 μL of DMSO, 0.5 μL of Phusion DNA polymerase (2 U/μL), and nuclease-free water to 50 μL.
3. Perform PCR with the reaction mix above in a thermocycler using the following PCR conditions to generate the amplicon F1-R1:

   Step 1 (1 cycle; initial denaturation).

   98 °C, 3 min

   Step 2 (30 cycles)

   98 °C, 15 s (denaturation).

   62 °C, 20 s (annealing at ~ Tm of primers).

   72 °C, 30 s per kb (extension).

   Step 3 (1 cycle: final extension).

   72 °C, 5 min
4. Using forward F2 and reverse R2 primers, perform PCR to amplify a 1-kb homologous sequence upstream of the CWSS (Fig. 1). Primer F2 contains a restriction enzyme site (XbaI), while primer R2 has the reverse complement of the overhang sequence (**step 1**) at its 5′-end. Use the same composition of the reaction mixture as mentioned in **step 2** and thermocycler conditions in **step 3** to generate F2-R2 (*see* **Note 2**).

3.2 Linking Amplified DNA Fragments by Overlapping PCR

1. Using a QIAquick gel extraction kit, purify the two PCR products (F1-R1 and F2-R2) above (see Subheading 3.1, **steps 3** and **4**) (*see* **Note 3**).
2. Measure DNA concentrations with NanoDrop 1000 spectrophotometer.
3. Set up a reaction mixture for the first part of overlapping PCR as follows: 4 μL of 5X Phusion GC buffer, 0.4 μL of 10 mM dNTPs, ~200 fmol each of F1-R1 and F2-R2, 1 μL of DMSO, 0.2 μL of Phusion DNA Pol (2 U/μL), and nuclease-free water (to 20 μL).
4. Perform PCR using the flowing conditions to generate a F2-R1 template from annealing and extending of F1-R1 and F2-R2 fragments:

   Step 1 (1 cycle).

   98 °C, 3 min.

   Step 2 (6 cycles)

   98 °C, 15 s.

   62 °C, 20 s.

   72 °C, 30 s per kb.

Step 3 (1 cycle).

72 °C, 3 min

5. Set up a reaction mixture for the second part of overlapping PCR as follows: 12 μL nuclease-free water, 4 μL of 5X Phusion GC buffer, 0.4 μL of 10 mM dNTPs, 1.2 μL each of 10 μM F2 and R1 primers, 1 μL of DMSO, 0.2 μL of Phusion DNA Pol (2 U/μL), and 20 μL of PCR products in **step 4**.
6. Perform PCR using the following conditions to amplify the F2-R1 product:

   Step 1 (1 cycle).

   98 °C, 2 min.

   Step 2 (30 cycles)

   98 °C, 15 s.

   62 °C, 20 s.

   72 °C, 30 s per kb.

   Step 3 (1 cycle).

   72 °C, 3 min
7. Purify the PCR product (F2R1) via gel extraction and measure DNA concentrations using a NanoDrop 1000 spectrophotometer.

3.3 Construction of Co-Integration Plasmids

1. Digest ~3 μg pHTTh plasmid DNA with HpaI and XbaI restriction enzymes in 1X CutSmart buffer for 2 h at 37 °C and purify by gel extraction.
2. Similarly, digest ~1.5 μg of the PCR product F2R1 above (Subheading 3.2, **step** 7) with XbaI restriction enzyme and purify (*see* **Note 4**).
3. Measure the DNA concentrations of the purified plasmid and the F2R1 fragment using a NanoDrop.
4. Set up a ligation reaction in a 0.1 mL PCR tube as follows: 75 ng of digested pHTTh plasmid, 75 ng of F2R1, 1 μL of 10X ligase buffer, and 0.5 μL of T4 DNA ligase, and nuclease-free water to 10 μL (*see* **Note 5**).
5. Mix the reaction by gently pipetting, incubate at 22 °C for 30 min, and inactivate at 75 °C for 5 min (*see* **Note 6**).
6. Mix 5 μL of the ligation mix above with 50 μL of *E. coli* DH5α competent cells (*see* **Note 7**), heat-shock cells for 30 s at 42 °C, and incubate on ice for 2 min. Add 500 μL of fresh LB broth prior to incubation at 37 °C for 1 h in a shaker incubator.
7. Pellet the cells by centrifugation at 10,000 x g for 60 s, resuspend the cell pellet in 100 μL of fresh LB broth, spread onto LB agar plates containing 50 μg/mL kanamycin, and incubate overnight at 37 °C. Extract plasmid DNA from positive clones (*see* **Note 8**), and measure its concentration.

3.4 Preparation of A. oris Competent Cells and Electro-Transformation at Room Temperature

1. Inoculate 3-mL cultures in HIB from a single colony of *A. oris* strains from HIA plates and grow in a water bath shaker at 37 °C overnight (O/N) (*see* **Note 9**).
2. Using the overnight cultures, prepare fresh HIB cultures (1:10 dilution) and grow at 37 °C with shaking until OD_{600} ~ 0.6.
3. Transfer 3.5 mL of the cultures to a sterile culture tube, add 1.5 mL of 15% glycine prepared in HIB (*see* **Note 10**), and incubate the tube in a water bath shaker at 30 °C for 1 h (*see* **Note 11**).
4. Harvest cell pellets from 1.5 ml of the above cultures by centrifugation at 8500 x g for 90 s, wash the pellets once with 1 mL of sterile dH_2O, and resuspend the pellets in 150 μL of sterile dH_2O (*see* **Note 12**).
5. Mix 150 μL of competent cells above with ~1 μg plasmid, incubate at room temperature for ~5 min, and transfer the mixture to a 2 mm gap electro-cuvette for electroporation at 2.5 kV.
6. Recover the cells with 1 mL of HIB at 37 °C for 2 h in an incubator with 5% CO_2 (*see* **Note 13**).
7. Pellet the cells by centrifugation at 9500 x g for 90 s (*see* **Note 14**), remove ~900 μL of the supernatant, and resuspend the cell pellet in the remaining 100 μL.
8. Spread on HIA plates containing 50 μg/ml kanamycin and incubate at 37 °C in an incubator with 5% CO_2 until co-integrant colonies appear (~3 days).

3.5 Selection of Integrant Mutants

1. Pick a colony from **step 8** above (Subheading 3.4) and streak on a fresh HIA plate containing kanamycin and grow overnight at 37 °C in an incubator with 5% CO_2.
2. Prepare a PCR reaction mix containing 4 μL of 5X Phusion GC Buffer, 0.4 μL of 10 mM dNTPs, 0.6 μL of 10 μM forward primer (F2), 0.6 μL of 10 μM reverse primer (R3), 1 μL of DMSO, 0.2 μL of Phusion DNA polymerase (2 U/μL), and nuclease-free water (to 20 μL) (*see* **Note 15**).
3. Using a sterile 200 μL tip, pick individual colonies from the above plate and rub them on the bottom of a sterile PCR tube containing 20 μl of the reaction mix above.
4. Perform colony-PCR to amplify the DNA region coding for the 1-kb fragment and mutated CWSS as follows:

Step 1 (1 cycle).

98 °C, 5 min.

Step 2 (30 cycles)

98 °C, 15 s.

62 °C, 20 s.

72 °C, 30 s per kb.

Step 3 (1 cycle).

72 °C, 5 min

5. Purify the PCR products by gel extraction and perform DNA sequencing to confirm desired mutations and the rest of the gene.

4 Notes

1. The overlapping sequence of primers F1 and R2 should be equal or greater than 18 bp long, but not too long to maintain the balance of melting temperature (Tm) and annealing capability. For bacterial genomes with high GC-content, like that of *A. oris*, the Tm of the primers should preferably be above 60 °C, and repeats of GGG should be avoided. Unless otherwise instructed, the primers should be designed such that the resulting sequences remain in-frame within the genome after co-integration.
2. To reduce the pipetting error, a 2X mastermix without genomic DNA and primers is preferably used; PCR reactions should be set up on ice for more specific amplification; and reaction volumes less than 50 μL yield better results.
3. PCR products are separated by using a 1% agar gel and cut from gels after they are fully resolved to prevent contamination from non-specific bands. Minimize UV exposure.
4. The insert is digested with only one enzyme XbaI since the other end is blunt. Note that roughly 30% of plasmid DNA is lost via gel extraction.
5. Keep the molar ratio of vector to insert as 1:3, which is calculated using Ligation Calculator (http://www.insilico.uni-duesseldorf.de/Lig_Input.html).
6. Do not vortex the ligation mix. Store it at −20 °C if not used immediately.
7. Do not use more than 5 μL ligation reaction for 50 μL competent cells.
8. Verify positive clones by colony PCR using any Taq DNA polymerase (such as Apex 2X RED Taq Master Mix) and

primers designed against the vector regions flanking the insert. The clones can be further validated by Sanger sequencing of the plasmid.

9. Use sterile metal loop for inoculation and immerse in sterile HIB before picking the cells from the plate.
10. 15% glycine in HIB should be prepared afresh. It can be stored at 4 °C for a few weeks.
11. Shaking speed and incubation time should not go beyond 160 rpm and 1 h, respectively. These conditions are critical.
12. Cells can be washed twice (optional). Be careful not to leave the cells in water for too long.
13. Fresh HIB media should be immediately added to electrocuvettes after pulsing, and the suspension is transferred to 1.5 mL Eppendorf tube for recovery.
14. Sometimes higher speed (~12,000 x g) is needed for pelleting.
15. Primer R3 is designed against the vector pHTTh.

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Chapter 11

Quantifying the Kinetics of Pilus-Specific Sortase-Catalyzed Crosslinking Using High-Performance Liquid Chromatography

Nicole A. Cheung, Mabel Song, Christopher K. Sue, and Robert T. Clubb

Abstract

Gram-positive bacteria display pili whose protein components (pilins) are covalently crosslinked by pilus-specific sortase enzymes. These cysteine transpeptidase enzymes catalyze a transpeptidation reaction that joins the pilins together via lysine isopeptide bonds. The crosslinking reaction that builds the SpaA pilus in *Corynebacterium diphtheriae* is mediated by the SrtA sortase (^{Cd}SrtA) and has been reconstituted in vitro. Here, we present a protocol that can be used to measure the kinetics of ^{Cd}SrtA-catalyzed crosslinking using high-performance liquid chromatography (HPLC). In principle, this biochemical procedure can be used to measure the in vitro crosslinking activity of any pilus-specific sortase.

Key words High-performance liquid chromatography, Sortase, Pili, Kinetics

1 Introduction

Many species of Gram-positive bacteria use pilus-specific sortase enzymes to build pili, long proteinaceous structures that extend from the cell surface to mediate bacterial adhesion. These cysteine transpeptidases catalyze a protein crosslinking reaction that joins the protein components (called pilins) of the pilus together via lysine isopeptide bonds [1–3]. Sortases are virulence factors in a range of clinically important pathogens and their enzymatic activities have been exploited to engineer proteins. Quantitative measurements of their bond-forming activities are therefore useful to gain deeper insight into their functions in pilus assembly, to facilitate the discovery of small molecule enzyme inhibitors and to further develop these enzymes as useful molecular biology tools. Here, we describe an HPLC-based method to measure the in vitro kinetics of lysine-isopeptide crosslinking activity. We use as an example the Sortase A enzyme from *Corynebacterium diphtheriae* (^{Cd}SrtA), which crosslinks SpaA pilins together to build the shaft of

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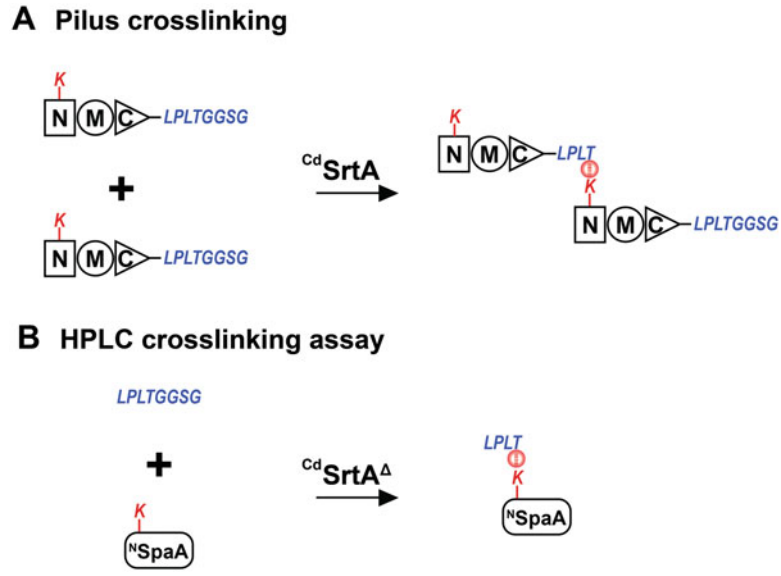

Fig. 1 Schematic of the SpaA crosslinking reaction catalyzed by ^{Cd}SrtA. (**a**) Crosslinking reaction used to build pili in which two SpaA proteins react. The SpaA protein contains: amino- (N, square), middle- (M, circle), and carboxy-terminal (C, triangle) domains. It also contains a cell wall sorting signal (blue) that follows the C-domain. The red circle containing the dashed line corresponds to the lysine isopeptide bond. (**b**) In vitro reconstitution of the crosslinking reaction using peptide and protein substrates. The N-domain of SpaA (^{N}SpaA) with the reactive lysine is attached to a peptide containing the LPLTG sorting signal peptide (residues FELPLTGGSG, called LPLT10). In the reaction, an activated sortase, ^{Cd}SrtA$^{\Delta}$, is used

the SpaA pilus [4–6]. In the transpeptidation reaction, ^{Cd}SrtA joins the LPLTG sorting signal located within the C-terminus of one SpaA pilin to the side of Lys190 located in a second SpaA pilin to create a K190(SpaA)-T494(SpaA) lysine isopeptide bond (Fig. 1a). To quantify the kinetics of this process the reaction is simplified, by using protein and peptide substrates (Fig. 1b). In the simplified reaction a soluble catalytic domain of ^{Cd}SrtA lacking its inhibitory "lid" structure is used (^{Cd}SrtA$^{\Delta}$, residues N37-Q257 of ^{Cd}SrtA in which the amino acids I78 to A88 are deleted). The kinetics of ^{Cd}SrtA$^{\Delta}$ -catalyzed crosslinking is then measured using two substrates, a polypeptide containing the N-domain of SpaA (^{N}SpaA) and a peptide containing LPLTG sorting signal (LPLT10, residues FELPLTGGSG) [7]. The method makes use of high-performance liquid chromatography and is a variation of the protocol that was developed to measure the activity of sortase enzymes that attach proteins to the cell wall [8]. While the *C. diphtheriae* ^{Cd}SrtA enzyme is used as an example in this biochemical procedure, in general this protocol can be employed for any pilus-specific sortase.

2 Materials

Ultrapure double-distilled Milli-Q water (ddH_2O) was used to prepare solutions, unless otherwise stated.

2.1 Crosslinking Reaction

1. Protein Buffer: 50 mM Tris-HCl and 300 mM NaCl, pH 8.0.
2. Purified Proteins: Both the $^{Cd}SrtA^{\Delta}$ sortase enzyme ($^{Cd}SrtA$, residues N37-Q257 in which residues I78 to A88 are deleted) and the protein substrate containing the reactive lysine ($^{N}SpaA$) are stored in Protein Buffer at 4 °C [5]. The stock solutions contained the $^{Cd}SrtA^{\Delta}$ and $^{N}SpaA$ proteins at concentrations of 500 μM and 1 mM, respectively (*see* **Notes 1** and **2**).
3. Sorting Signal Peptide (Sequence FELPLTGGSG, referred to herein as the LPLT10 peptide). It is dissolved in Protein Buffer to a concentration of 20 mM (*see* **Notes 3** and **4**).
4. 1 M dithiothreitol (DTT).
5. Liquid N_2, or dry ice and ethanol (for flash freezing).
6. 1.5 mL Eppendorf tubes.
7. Rocker or rotator.
8. Incubator at temperature of choice (required for reactions that are not performed at room temperature).

2.2 HPLC Assay

1. Buffer A: 100% water with 0.1% trifluoroacetic acid (TFA). Add 1 mL of TFA to 999 mL deionized water, then filter with 0.22 μm nylon filters and degas by sonication or vacuum filtration (*see* **Note 5**).
2. Buffer B: 100% acetonitrile with 0.1% TFA. Add 1 mL of TFA to 999 mL Acetonitrile, then filter with 0.22 μm nylon filters and degas by sonication or vacuum filtration (*see* **Note 5**).
3. HPLC instrument: All experiments shown were performed using an Agilent 1100 HPLC system.
4. HPLC Column: Symmetry 300TM C4 5 μM (Waters-Part No: 486000288). Store in 100% acetonitrile (*see* **Note 6**).
5. Nylon-filter polypropylene chromatography vials (Agilent-Part No: 5610-2119).

3 Methods

3.1 Crosslinking Reactions

All reactions are carried out at room temperature (25 °C); however, the protein reagents and DTT should be kept cold on ice before adding them to the reaction mixtures.

1. Set up 100 μL reactions in Eppendorf tubes with appropriate reagent concentrations for the purpose of the experiment. For kinetic measurements, the final reaction concentrations are as follows: 25 μM $^{Cd}SrtA^{\Delta}$, 1 mM LPLT10 peptide, varying serially diluted concentrations of $^{N}SpaA$ (500–62.5 μM), and 5 mM DTT in aqueous solution. Add Protein Buffer for the remaining volume up to 100 μL. Reagents can be added in any order, as long as the $^{Cd}SrtA^{\Delta}$ enzyme is added last, since it will initiate the reaction. Place the samples on a rocker or rotator during incubation.
2. Remove a 10 μL sample from the reaction and place it in an HPLC-compatible vial. Samples are acquired 0, 1, 3, 6, 12, and 24 h after initiating the reaction (*see* **Note 7**). Samples are immediately flash frozen by immersing them in liquid N_2 or a dry ice/ethanol bath (*see* **Note 8**). Use tongs to immerse the sample tube into the bath until the portion of the tube containing the sample is submerged and the liquid sample is thoroughly frozen. Store the sample at −20 °C until you are ready to perform the HPLC experiments.

3.2 HPLC Experiments

The goal is to use HPLC to separate the reaction components such that their abundance can be quantified by analyzing the chromatogram. This will require using the appropriate column and gradient conditions to separate the components. For the $^{Cd}SrtA^{\Delta}$ reaction described in this protocol the following method is used (*see* **Note 9**).

Time (min)	% Buffer A	% Buffer B	Flow rate (mL/min)
0	64	36	1
3	64	36	1
13	56	44	1
14	0	100	3
16.3	0	100	3
17.3	64	36	3
19.6	64	36	3

1. Configure the HPLC instrument with the C4 column and solvents that have been filtered and degassed (refer to Subheading 2.2 for additional details on solvent filtering and degassing). For the Agilent 1100, ideally 1.0 L of each solvent should be connected to the instrument, but this reservoir amount is unique to each instrument (*see* **Note 10**).

2. Run control experiments to ensure that the instrument is working properly and to assist in subsequent data analysis. In control experiments, several samples will be injected separately and the gradient method will be applied. The injections are 5 μL. In the first control experiment, only buffer A is injected. The absence of significant eluate peaks in the chromatogram is evidence that the column is clean and can be used to separate the reactants. In the next set of control experiments, separate injections are performed using each of the three reactants: $^{Cd}SrtA^{\Delta}$, $^{N}SpaA$, and the LPLT10 peptide. In these experiments, each reactant should eluate at ~8 min, ~14 min, and ~2 min, respectively (time after the injection). When overlaid, the peaks corresponding to these reactants should look similar to the chromatogram shown in Fig. 2a.
3. Having ensured the instrument is working properly, use the HPLC to analyze the samples from the reactions described in Subheading 3.1. In this process, one frozen sample from the reaction assay is thawed and injected into the HPLC column for analysis.
4. Repeat the process outlined in **step 3** for all samples in the reaction time course (*see* **Notes 11** and **12**).

3.3 Data Analysis: Estimating the Steady-state Enzyme Kinetics

Estimates of the steady-state enzyme kinetics parameters can be obtained by measuring the reaction velocity at different substrate concentrations (either varied LPLT10 or $^{N}SpaA$ substrate concentrations). For example, in this protocol the kinetic parameters are obtained by varying $^{N}SpaA$ from 500 μM to 62.5 μM (*see* **Note 13**). Typically, a two time point measurement of product formation is used to determine the velocity (0 and 3 h). The dependence of the reaction velocities on substrate concentration is then used to determine the K_M and k_{cat} parameters.

1. Analyze the chromatograms obtained for each reaction time point. This is done by integrating the peaks that correspond to the $^{N}SpaA$ reactant and the $^{N}SpaA$-LPLT product in the chromatogram (*see* **Note 14**). The area of the peak is linearly proportional to the amount of each species that is present.
2. Calculate the concentration of the product that is formed after 3 h. Initially, the areas of the substrate and product peaks are used to calculate the fraction of protein substrate that is converted into the modified protein product. This corresponds to the area of the product peak divided by the sum of the $^{N}SpaA$-LPLT product and $^{N}SpaA$ substrate peak areas. The concentration of the modified $^{N}SpaA$-LPLT protein product is then determined by multiplying this fraction by the concentration of the substrate that was initially present in the reaction.

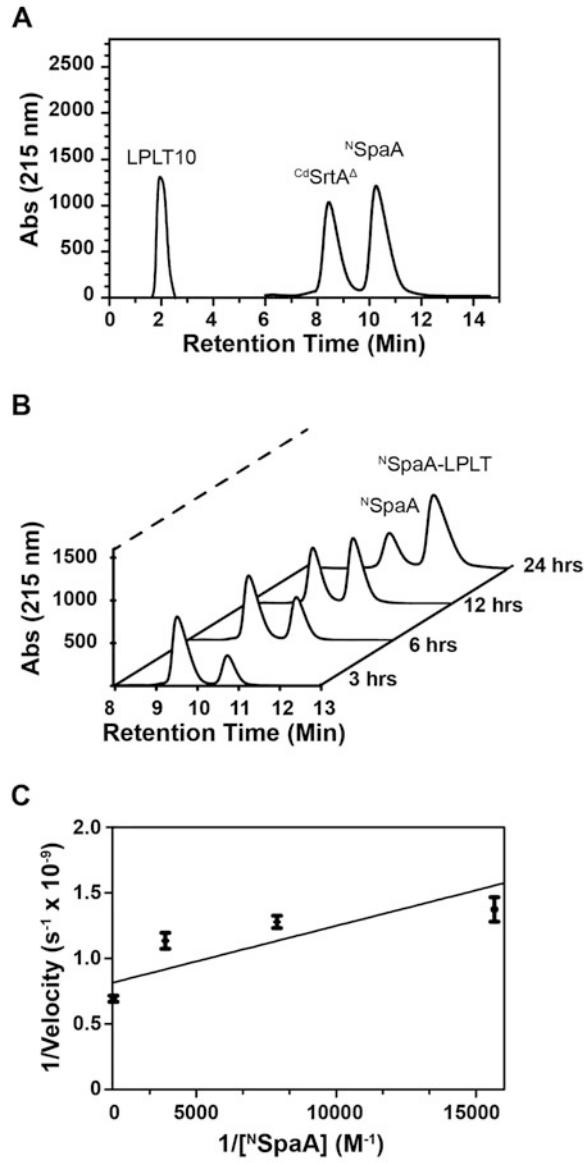

Fig. 2 Example HPLC chromatograms for the sortase-catalyzed crosslinking reaction. (**a**) Chromatogram obtained immediately after mixing the reactants (0 h elapsed). The figure shows good separation of the sortase (^{Cd}SrtA$^{\Delta}$), protein (^{N}SpaA), and sorting signal peptide (LPLT10). (**b**) Offset and overlaid chromatograms of the reaction time course showing the region that contains ^{N}SpaA and the ^{N}SpaA-LPLT10 product. (**c**) Sample double-reciprocal Lineweaver-Burk plot of the kinetics data that can be used to estimate the steady-state kinetic parameters

3. Estimate the velocity for each substrate concentration. The product formed should increase in a linear fashion as a function of time. If this is the case, the velocity can be calculated by dividing the concentration product formed by the amount of time elapsed (units of M/s) (for the $^{Cd}SrtA^{\Delta}$ reaction described in this protocol, the product formed after 3 h is used to estimate the velocity).
4. Since reaction velocities are determined for only a few substrate concentrations, a Lineweaver-Burk plot is used for the analysis. To obtain a Lineweaver-Burk plot, create a graph of the reciprocal of the initial velocity as a function of reciprocal substrate concentration (Fig. 2c).
5. Use software to fit the graph to extract the steady-state kinetic parameters. We typically fit our data using SigmaPlot 12.0 or MATLAB programs.
6. The maximum velocity (V_{max}) is represented by the reciprocal of the *y*-intercept ($1/V_{max}$).
7. The Michaelis constant (K_M) represents the substrate concentration at which the reaction rate is half the maximum velocity. The slope of the fitted plot is equal to K_M/V_{max}.
8. The first-order rate constant (k_{cat}) can be calculated from the maximum velocity (V_{max}) divided by the enzyme concentration.

4 Notes

1. Protein concentrations were quantified using UV absorbance and the molar extinction coefficient of the proteins, or by using a Bradford Assay.
2. $^{Cd}SrtA^{\Delta}$ is a mutated variant of $^{Cd}SrtA$ in which the lid region that covers the active site is removed. This assay will also work using $^{Cd}SrtA^{2M}$ and $^{Cd}SrtA^{3M}$ enzyme variants, which also disable the lid [6, 7]. It could also be used to study the activity of pilus-specific enzymes from other microorganisms.
3. Peptides should be stored as recommended by the manufacturer.
4. Peptides in their powdered form can have different solubilities depending on their size and hydrophobicity. Consult the peptide manufacturer for details on how to properly solubilize and store your peptide.
5. Nylon filters are essential. Other types of filters will be degraded by the TFA.
6. The choice of the column used for separation is system specific. For proteins with varying hydrophobicity, sometimes a C8

column may be preferable. For small peptides, a C18 column may be preferred.

7. The time points for sample collection will depend on the enzyme system. If applying this method to other reactions, shorter time points may need to be collected if the enzyme being studied is more active than $^{Cd}SrtA^{\Delta}$.
8. Flash freezing is not essential. If you would like the samples to be run immediately, plan to run the blank sample immediately before taking the desired time point sample, such that the assay samples can be run consecutively without interruption.
9. The method shown in Subheading 3.2 is optimized for $^{Cd}SrtA^{\Delta}$. The parameters used for the gradient method will depend on the enzyme-substrate system and must be optimized.
10. For a peptide bond, the detection wavelength is typically 215 nm. If the protein contains Tyr or Trp residues absorbance at 280 nm can be used.
11. Different enzymes may require different methods and columns. Each method requires its own blank to be equilibrated properly. If you are using multiple methods, the respective blank must precede the experiment.
12. For an Agilent 1100 instrument ensure that the next sample is loaded prior to the completion of the previous sample's run. Otherwise, the warm-up and blanking process will have to be repeated.
13. It is important to emphasize that only approximate values for K_M and k_{cat} are determined. The reaction contains two substrates. The concentration of one substrate is varied, while the other substrate is present at saturating concentrations (in the protocol the LPLT10 peptide is saturating and $^{N}SpaA$ is varied). If the second reactant is not at saturating concentrations then the maximal velocity cannot be achieved. Therefore the k_{cat} value will be underestimated. This is the case for the $^{Cd}SrtA^{\Delta}$ -catalyzed reaction.
14. This can be done using the instrument software (Agilent ChemStation for the Agilent 1100). It can also be done by exporting the data and analyzing with other software programs such as Empower, Chromeleon, and OpenLab. The $^{N}SpaA$ reactant and the $^{N}SpaA$-LPLT product are assumed to have nearly identical extinction coefficients at 215 nm.

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Chapter 12

Detection of Cell Wall-Anchoring Machinery by Immunogold-Labeling Thin-Section Electron Microscopy

Chungyu Chang and Hung Ton-That

Abstract

Cell wall anchoring of surface proteins and pili in Gram-positive bacteria is mediated by sortase – a highly conserved transpeptidase enzyme. Early studies have demonstrated the membrane-associated nature of this enzyme in close proximity with its cognate substrates, using immunogold-labeling thin-section electron microscopy. Here, we provide a detail protocol of this methodology, including specimen preparation, ultrathin sectioning, and immunogold-labeling electron microscopic procedures, with an experimental model of sortase enzymes from *Actinomyces oris*. In principle, this protocol can be employed for any bacterial ultrathin-section samples to detect subcellular localization of proteins and organelles by immuno-electron microscopy.

Key words Electron microscopy, Immunogold labeling, Thin-section, Sortase, Cell wall anchoring, Pili

1 Introduction

In Gram-positive bacteria, covalent attachment of surface proteins and adhesive pili to bacterial peptidoglycan requires a conserved transpeptidase enzyme often referred to as the housekeeping sortase [1]. Many Gram-positive bacteria, such as *Corynebacterium diphtheriae*, *Actinomyces oris*, *Bacillus cereus*, *Enterococcus faecalis*, and *Streptococcus agalactiae*, possess additional copies of specialized sortase enzymes, i.e., class C sortases, which catalyze pilus polymerization, bonding individual pilus proteins into covalently linked pilus polymers [2–6]. These polymers are subsequently anchored to the bacterial cell wall by the housekeeping sortase [4, 7–10]. As sortases are membrane bound, it has been shown that sortase enzymes, their cognate substrates, and SecA – the ATPase motor of the secretion machine – are co-localized within microdomains of the cytoplasmic membrane, as revealed by immunogold-labeling ultrathin-section electron microscopy [11, 12]. Here, using *A. oris*

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as an experimental model, we provide a detailed protocol of this approach to detect membrane-bound sortase enzymes – the housekeeping sortase SrtA and the pilus-specific sortase SrtC2; the latter catalyzes formation of type 2 fimbriae consisted of the tip fimbrilin CafA and the shaft pilin FimA [13]. This protocol involves sequential labeling of thin-section samples with two antibodies and IgG-conjugated gold particles of different sizes. Although two antibodies independently raised from dissimilar animal species, e.g., rabbit and mouse, are preferred, this protocol can utilize antibodies raised from the same species, e.g., rabbit or mouse. While sortases are used as an example in this microscopic procedure, in general this protocol can be employed for any bacterial ultrathin sections to detect subcellular localization of proteins and organelles by immuno-electron microscopy.

2 Materials

Most materials used for thin section preparation were purchased from Electron Microscopy Sciences (EMS) (http://www.emsdiasum.com/microscopy/). Ultrapure double-distilled Milli-Q water (ddH_2O) was used to prepare solutions, unless otherwise stated.

2.1 Bacterial Specimen Preparation

1. Media: heart infusion broth (HIB) and heart infusion agar (HIA) (1.5% agar in HIB).
2. Water bath shaker, 37 °C.
3. Eppendorf tubes (Epp tubes).
4. Spectrophotometer.
5. Microfuge.
6. Platform rocker or rotator.
7. 64 °C incubator.
8. Sterile ddH_2O.
9. 0.2 M and 0.1 M Trihydrate Sodium Cacodylate (Na cacodylate) (EMS) pH 7.4 (*see* **Note 1**).
10. 4% Low melting point (LMP) agarose in ddH_2O.
11. Dental wax (EMS).
12. Razor blades.
13. 200 mesh formvar/carbon/nickel grids (EMS).
14. Dumont tweezers #5.
15. Dumont tweezers N4AC (EMS).
16. Parafilm.

17. Petri dishes (square shape is recommended).
18. Pelco easiGlow™ discharger.

2.2 Chemicals for Fixation, Dehydration, Embedding Resin

1. 16% Paraformaldehyde, EM grade (EMS).
2. 25% Glutaraldehyde, EM grade (EMS).
3. Freshly prepared fixation solution: 5 mL of 16% paraformaldehyde, 0.08 mL of 25% glutaraldehyde, 10 mL of 0.2 M Na cacodylate buffer, and 4.92 mL of ddH_2O.
4. Ethanol (EtOH, 200 proof).
5. Freshly prepared 50% EtOH and 70% EtOH solutions.
6. Embedding resin: LR White (EMS).
7. Freshly prepared dehydration solutions: 50% LR White/50% EtOH and 75% LR White/25% EtOH.
8. BEEM capsule (EMS).
9. BEEM capsule holder (EMS).
10. Polyethylene pipets.
11. Dehydration vials.

2.3 Chemicals for Immuno-gold Labeling

1. Aurion Blocking Solution (EMS, for antiserum made from rabbit; check the EMS website for blocking solutions suitable for various sources of antisera).
2. Aurion BSA-c™ 10% solution (EMS).
3. PBS (10 mM phosphate buffer, pH 7.4, 150 mM NaCl); sterile-filter before use.
4. 50 mM Glycine: Dissolve 37.3 mg in 10 mL PBS; sterile-filter before use.
5. Incubation buffer: 0.1% Aurion BSA-c™ in PBS (*see* **Note 2**).
6. Colloidal gold conjugated secondary antibody, 12 nm or 18 nm (*see* **Note 3**).
7. 1% uranyl acetate (EMS): Dissolve 100 mg uranyl acetate in 10 mL of ddH_2O.

3 Methods

3.1 Specimen Preparation and Fixation

1. Prepare late-log phase *A. oris* cell cultures in heart infusion broth (HIB), with OD_{600} ~ 0.7–0.8.
2. Spin down 1 mL of the cultures in an Epp tube at 8000 × *g* for 2 min and wash the cell pellet gently (no vortexing) once by suspending in 1 mL of 0.1 M Na cacodylate buffer, pH 7.4.
3. Resuspend the washed cell pellet in 50 μL of 0.1 M Na cacodylate buffer (1:1 dilution from 0.2 M stock).

4. Prepare 2% LMP agarose by mixing 1 mL of 4% LMP agarose melt in sterile ddH_2O and 1 mL of 0.2 M Na cacodylate buffer (prewarmed before use) and incubated at 42 °C before use.
5. Add 50 μL of 2% LMP agarose slowly to the cell suspension in **step 3** and mix them gently (no air bubbles). Let the mixture become completely solidified at room temperature.
6. Flush out the solidified agarose from the Epp tube by adding 500 μL of Fixation solution (*see* **Note 4**).
7. Dice the solidified mixture (**step 5**) into 1 mm cubes with the razor blade on a sheet of dental wax. Transfer the samples to dehydration vials (20–30 cubes per vial).
8. Soak the 1 mm cubes in 10–15 mL of 3% EM grade paraformaldehyde fixing solution (pH 7.3–7.4) at 4 °C overnight in a dehydration vial (*see* **Note 5**).
9. Remove the fixation solution from with polyethylene pipets before adding 10 mL 0.1 M cacodylate buffer, pH 7.3–7.4, to wash the samples. Each wash lasts 10 min for three times (3 × 10 min) (*see* **Note 6**).
10. Wash samples with 10 mL of ddH_2O for 15 min twice (2 × 15 min), note: do not post-fix in osmium tetroxide (*see* **Note 7**).

3.2 Preparation of Ultrathin Sections

1. Dehydrate the samples by sequentially changing dehydration solutions with 10 mL in each step using the following order:
   (a) 3 × 5 min with 50% EtOH
   (b) 3 × 5 min with 70% EtOH
   (c) 2 × 2 h with 50% LR White/50% EtOH
   (d) 4 h with 75% LR White/25% EtOH
2. Change the above samples to 15 mL of 100% LR White and gently rock the vials on a platform shaker overnight at room temperature.
3. Change to fresh 15 mL of 100% LR White in the morning and incubate the vials for another 6 h.
4. Transfer the sample cubes into BEEM capsules and fill up the capsules below the edge with LR White. Cure the BEEM capsules in an incubator at 64 °C while they rest on the capsule holder (*see* **Note 8**).
5. Prepare ultrathin sections with 60–80 nm thickness by a Diatome diamond knife on 200-mesh formvar/carbon/nickel grids (*see* **Note 9**).

3.3 Immuno-Gold Labeling

3.3.1 Single Labeling (Fig. 1a)

1. Prepare individual 500 µL-drops of 50 mM glycine in PBS on a piece of parafilm placed inside a petri dish (*see* **Note 10**).
2. Float a thin section-containing grid upside down on a 500 µL-drop inside the covered petri dish for 15 min at room temperature. Note: Subsequent steps are followed the same way. It is extremely important not to let the grid sink to the bottom of the droplet.
3. Wash the grids twice on two drops of PBS by gently pipetting the buffer underneath the grid up and down for ~60 times before transferring the grid to the next drop of buffer (*see* **Note 11**).
4. Block the grids onto one drop of Aurion Blocking Solution for 30 min (*see* **Note 12**).
5. Wash the grids three times on three drops of freshly made incubation buffer (0.1% Aurion BSA-c™ in PBS). The grids are washed in the same manner as described in **step 3** as well as for the following **steps 8, 10, 11** and **13**.
6. Dilute the primary antibody in Incubation Buffer (*see* **Note 13**).
7. Incubate the girds on a drop of 100 µL diluted antibody for 2 h at room temperature (*see* **Note 14**).
8. Wash 6 times on six drops of Incubation Buffer.
9. Incubate the grid for 2 h at room temperature with the secondary antibody conjugated with gold particles with 1:100 dilution in Incubation Buffer.
10. Wash 6 times on six drops of Incubation Buffer (see below for double labeling).
11. Wash 3 times on three drops of PBS.
12. Postfix the grids with 2% glutaraldehyde in PBS for 5 min.
13. Wash 2 times with PBS, then 5 times with ddH_2O.
14. Hold the grid using the Dumont tweezers N4AC and then drop 7 µL of 1% uranyl acetate for contrast-staining for 2 min.
15. Wig-dry from the side of the grid with Kimwipes and flip the grid upside down on a piece of Kimwipes to immediately dry the grid.

3.3.2 Double Labeling (Fig. 1b, c)

For double labeling, the first five steps are the same as those in single labeling above. If two primary antibodies were raised from different animal species, e.g., rabbit and mouse, both antibodies can be mixed and incubated with the grids as described in **steps 6–7** above and proceed to **step 8**. In **step 9**, the grids are incubated with two species-specific antibodies conjugated with different sizes of gold particles. If both primary antibodies were produced with the same species, e.g., rabbit or mouse, complete **steps 6–10** with one

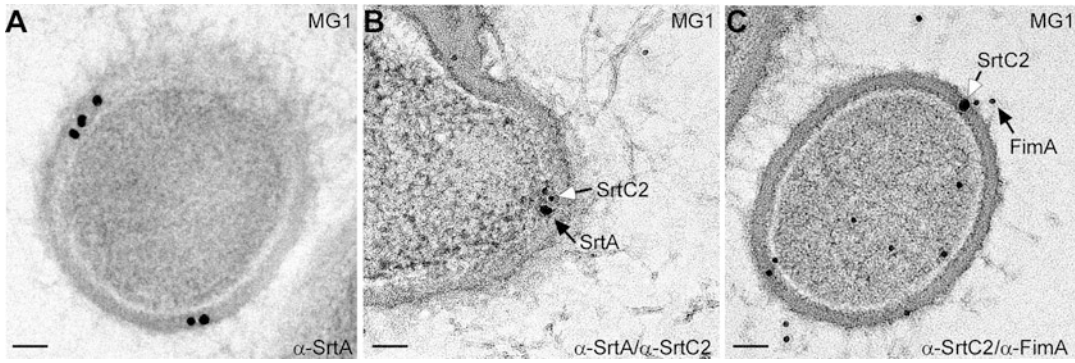

Fig. 1 Detection of cell wall-anchoring machinery by thin-section electron microscopy. (**a**) Nickel grids containing 55-nm ultrathin sections of *A. oris* MG1 cells were incubated with polyclonal antibodies against SrtA (α-SrtA) at 1:50 dilution, followed by anti-rabbit secondary antibody conjugated with 18 nm gold particles at 1:100 dilution. The samples were stained with 1% uranyl acetate prior to viewing with an electron microscope. A scale bar indicates 50 nm. (**b**, **c)** Like (**a**), ultrathin sections of *A. oris* MG1 cells were double-labeled with antibodies against SrtA and SrtC2 and 18-nm and 12-nm gold particles conjugated to IgG, respectively (**b**) or with antibodies against SrtC2 and FimA and 18-nm and 12-nm gold particles conjugated to IgG, respectively (**c**); scale bar of 50 nm

primary antibody followed by floating the grids in the second primary antibody for 2 h (**step 7**). Complete **steps 8–15**; note: in **step 9**, use a second antibody conjugated to gold particles with a different size from the first one.

4 Notes

1. 0.2 M trihydrate sodium cacodylate stock: 8.56 g of Na cacodylate +200 mL ddH_2O, using HCl to adjust pH of 7.36.
2. Use 100 μL of 10% Aurion BSA-c™ in 9.9 mL of PBS to make 0.1%.
3. Depending on the source of the primary antibody, if the antibody was generated from rabbits, use an anti-rabbit colloidal-gold conjugated secondary antibody.
4. To flush the 100 μL solidified agarose from the bottom of the tube, add 100 μL fixation solution first to lubricate and separate the agarose from the tube wall with a 10 μL pipette tip, then add 400 μL of the remaining fixation solution. The solidified agarose will float in the solution, which can be picked up gently with a pair of tweezers.
5. The lower the concentration of glutaraldehyde, the higher the antigenicity yields.
6. The sample vials are gently rocked on a platform shaker during washing and the rest dehydration processes.

7. To preserve the antigenicity, avoid osmium tetroxide.
8. Time-manage the entire experiment so that embedding occurs on a weekday and the 12–18 h curation ends before weekend.
9. It is recommended to use nickel grids, since copper grids react badly with the immuno-gold reagent. The grids should be glow-discharged (Pelco easiGlow™, 15 mA, 1.5 min) before use to increase the hydrophilicity to pick up the floating thin sections from underneath.
10. This treatment step is to inactivate the residual aldehyde groups from fixation.
11. This type of wash allows the grid to move freely on the top of drop and buffer exchange. It also removes nonspecifically bound molecules efficiently. It is recommended not to blow air bubbles during this wash.
12. Depending on the source of the antiserum generated from, choose the appropriate Aurion Blocking Solution according to the EMS website.
13. Depending on the titer of the antibody, different dilutions can be applied; a control grid without primary antibody in the buffer (mock) is recommended.
14. If the antiserum is low titer, incubate the grid overnight at 4 °C. Make sure the drop of antibody does not get dry by placing a piece of wet Kimwipes inside the petri dish.

Acknowledgments

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Chapter 13

Localization of the Remnant of a Cell Wall Sorting Signal and Its Interaction with a Sensor Kinase

Jeffrey W. Hall, Bruno P. Lima, Karen F. Johnstone, and Mark C. Herzberg

Abstract

Sortases are highly conserved enzymes with endopeptidase and transpeptidase activities in Gram-positive bacteria. Sortase A cleaves within an LPXTG-motif and covalently crosslinks cell wall proteins to become anchored to the peptidoglycan of the cell wall. We showed that a peptide cleaved by sortase A from the C-terminus (C-pep) of the LPXTG-adhesin SspA intercalates in the cell membrane. Nested in the membrane, this C-pep docks with the intramembrane sensor histidine kinase, SraS, to activate the response regulator, SraR. SraR signals that the C-pep has been cleaved as an indicator of the fidelity of sortase A processing. SraSR also signals that key LPXTG-proteins in concert with lipoteichoic acid engage the mucin, MUC5B, which elicits a different transcriptional response than the binding of other salivary constituents. To visualize the C-pep intercalating in the cell membrane in vivo, we used Structured Illumination Microscopy (SIM). And to show that the C-pep complexes with SraS, we used bimolecular fluorescence experiments. The C-pep and SraS were each expressed with one or the other half of yellow fluorescence protein (YFP). Reconstitution of the complete YFP signal indicated that the C-pep and SraS interacted at molecular distances within the cell membrane in vivo. Using these imaging protocols, we learned that the C-pep functions as a signaling molecule within the cell membrane of the streptococcal cell.

Key words Structured illumination microscopy, bimolecular fluorescence, Sortase A

1 Introduction

Current advances in imaging techniques facilitate the subcellular visualization of labeled peptides and interactions with proximal proteins. These imaging techniques have contributed to our understanding of subcellular signaling mechanisms in *Streptococcus gordonii*. One such mechanism described recently shows how the cell ascertains the fidelity of cell wall protein processing by sortase A. Sortase A cleaves a C-terminal peptide (C-pep) from LPXTG-motif proteins. First, to show that the C-pep cleaved by sortase A localizes in the streptococcal cell membrane, we used structured illumination microscopy (SIM) [1]. For this approach, the cleaved C-terminal peptide is fluorescently labeled in vivo and is visualized

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as co-localized with the lipid-stained cell membrane. To show that the peptide docks with the intramembrane sensor histidine kinase, SraS, we used bimolecular fluorescence (BiFC) [1]. SraS was expressed on the same message with one-half of yellow fluorescent protein (YFP I152L) and the C-pep expressed with the other half [2]. Gain of the YFP fluorescent signal in the cell marks the close interaction between the C-pep and SraS. Using these methods, we addressed a long-standing question of the fate and function of the sortase A-cleaved C-terminal peptide [3] and discovered a previously unrecognized outside-in signaling pathway in *S. gordonii* that may play a novel role in mucosal colonization in polymicrobial communities [4]. These methods, as we describe, may be widely applicable to visualizing subcellular localization of peptides and proteins and their functional interactions within cells.

2 Materials

2.1 Bacterial Culture Preparation

1. The construction and validation of the *S. gordonii* strains used in this protocol are described in Hall et al. 2019, Science Signaling (*see* **Note 1**).
2. Incubator with 5% CO_2 atmosphere, 37 °C.
3. 15 mL conical vials, sterile.

2.2 SIM Sample Preparation

1. X-15R centrifuge, SX4750 rotor (Beckman Coulter).
2. 1.7 mL sterile microfuge tubes.
3. Bench top vortexer.
4. 25 mm round glass coverslip #1.5, pre-cleaned.
5. TetraSpeck™ Microspheres 0.1 μm (ThermoFisher Scientific).

2.3 Bimolecular Fluorescence Specimen Preparation

1. 96-well polystyrene, non-tissue culture-treated plate with a clear flat bottom and black sides.
2. Microplate spectrophotometer with a GFP filter (485/540 nm) selected and tunable or selectable wavelength filter set at $\lambda = 600$ nm.

2.4 Instrumentation

1. Structured illuminated microscopy (SIM) was performed using a homemade system built essentially as described [5, 6].

2.5 Chemicals

1. Brain heart infusion broth (BHI).
2. FM® 4-64FX.
3. Hoechst 33342.
4. ProLong® Diamond.
5. Paraformaldehyde, 4% in PBS diluted 1:4 in PBS, pH 7.4.
6. Phosphate buffered saline, PBS, pH 7.4, sterile.

3 Methods

3.1 Specimen Preparation and Fixation

1. Culture, from an agar stab or frozen glycerol stock, *S. gordonii* SGO_0707-Venus YFP I152L (SGO_0707-YFP) statically in 10 mL of BHI overnight at 37 °C with a 5% CO_2 atmosphere using a sterile 15 mL conical vial.
2. Dilute 1 mL of the SGO_0707-YFP culture into 9 mL of sterile BHI broth in a 15 mL conical vial containing FM® 4-64FX (15 ng/μL) and incubate the culture for 4 h at 37 °C with a 5% CO_2 atmosphere.
3. Centrifuge the SGO_0707 YFP culture at 4 °C and 4750 RPM for 10 min to pellet the cells.
4. Using the centrifugation protocol in **step 3**, wash and resuspend the cells twice with 10 mL ice-cold PBS, pH 7.4 and pellet the cells a final time.
5. Resuspend the washed cell pellet in 1 mL of freshly made 1% paraformaldehyde-PBS, pH 7.4 supplemented with 20 μg/mL Hoechst 33342.
6. Incubate the cells for 10 min at 4 °C.

3.2 SIM Sample Preparation and Imaging

1. Vortex and remove 15 μL of cells and place in a fresh microfuge tube.
2. Working quickly, vortex the 0.1 mm TetraSpeck™ Microspheres and remove 5 μL of the microspheres and mix them with the cells.
3. Using a pipette, gently mix and then remove a ~20 μL volume and spread the mixture gently and evenly on 25 mm #1.5 glass coverslip.
4. Allow the mixture to dry on the coverslip in a dark environment.
5. Once dry, add enough ProLong® Diamond Antifade Mountant to cover the dried sample.
6. Place a second clean glass coverslip over the first coverslip. Allow the mountant to cure for 24 h at room temperature in a dark environment.
7. Acquire images using SIM with the following excitation laser lines and emission filters.
   (a) YFP: 488 nm laser line, GFP filter (BP 525/45).
   (b) FM 4-64FX 561 nm laser line, far-red filter (LP 635).
   (c) Hoechst 33342 405 nm laser line, DAPI filter (BP 480/40).
8. Data acquisition and post-processing was performed as described [6].

3.3 Bimolecular Fluorescence (BiFC) Signal Acquisition

1. Using agar stabs or frozen glycerol stocks, culture all the single and double recombinant BiFC *S. gordonii* strains statically in 10 mL of BHI at 37 °C with a 5% CO_2 atmosphere overnight (~16 h).
2. The following day, in triplicate, aliquot 200 μL of sterile BHI (same lot as the day before) into the A1-3 wells of a polystyrene 96-well, non-tissue culture treated plate with a clear flat bottom and black sides.
3. Vortex and aliquot 200 μL of each strain in triplicate into the microplate starting at B1-3 and working from top to bottom, left to right.
4. Load the microplate into a microplate reader with a GFP filter selected and wavelength filter set at $\lambda = 600$ nm.
5. Read the optical density ($OD_{600\ nm}$) and fluorescence signal of all samples.
6. Repeat with fresh cultures to obtain biological replicates for data analysis.

3.4 Bimolecular Fluorescence Data Analysis

1. Using appropriate software (microplate reader (i.e., Gen5, Magellan, etc.), or data analysis software (i.e., Microsoft Excel)), calculate the mean $OD_{600\ nm}$ and GFP fluorescence signal for all strains from each technical replicate for each biological replicate ((i.e., Sample 1 + Sample 2 + Sample 3)/3 = mean sample signal).
2. Blank the mean $OD_{600\ nm}$ and GFP signals by subtracting the respective mean signals of the sterile BHI from each of the sample means
   (a) Sample mean $OD_{600\ nm}$ – BHI mean $OD_{600\ nm}$ = Blanked sample mean $OD_{600\ nm}$
   (b) Sample GFP intensity – BHI mean GFP intensity = Blanked sample mean GFP intensity
3. Calculate the relative fluorescence units (RFU) for each sample by dividing the sample mean GFP signal by the sample mean $OD_{600\ nm}$ (sample mean GFP/sample mean $OD_{600\ nm}$ = RFU).
4. Using an appropriate statistical software package, analyze the data across biological replicates using a one-way ANOVA to identify protein interactions that reconstitute the full-length YFP protein and restore fluorescence capability to the complex as compared to single domain strains (*see* **Note 3**).

4 Notes

1. YFP-I152L, a halide-sensitive variant of yellow fluorescent protein (YFP), was chosen for its reduced background fluorescence, enhanced signal-to-noise and (S/N) ratio, and previous validation in BiFC experiments [2]. VN155 and VC155 fragments were utilized in all possible single and double-mutant formats.
2. Preparation of multiple coverslips for SIM is recommended.
3. Single-domain strains should have very low RFU signals. Moderate to high RFU levels in single-domain mutants suggest protein auto-aggregation and auto-fluorescence, which should be further investigated and resolved. In this experiment, the SGO_0707-YFP strain was used as positive control for YFP fluorescence detection.

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Chapter 14

Determination of the Crystal Structure of the Cell Wall-Anchored Proteins and Pilins

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Abstract

Surface proteins and pili (or pilus) anchored on the Gram-positive bacterial cell wall play a vital role in adhesion, colonization, biofilm formation, and immunomodulation. The pilus consists of building blocks called pilins or pilus subunits. The surface proteins and pilins share some common sequences and structural features. They contain an N-terminal signal sequence and the C-terminal cell wall sorting region, enabling their transportation across the membrane and covalent attachment to the bacterial cell wall, respectively. The transpeptidase enzymes called sortases facilitate the covalent links between the pilins during the pilus assembly and between surface proteins or basal subunits of pili and peptidoglycan-bridge during the cell wall anchoring. Thus, elucidating three-dimensional structures for the surface proteins and pilins at the atomic level is essential for understanding the mechanism of adhesion, pilus assembly, and host interaction. This chapter aims to provide a general protocol for crystal structure determination of surface proteins and pilins anchored on the Gram-positive bacterial cell wall and substrates for sortases. The protocol involves the production of recombinant protein, crystallization, and structure determination by X-ray crystallography technique.

Key words Pili, Pilins, Sortase, Surface proteins, Purification, Crystallization, X-ray crystallography

1 Introduction

Gram-positive bacteria use their cell wall-anchored surface proteins (e.g., MSCRAMMs – microbial surface components recognizing adhesive matrix molecules) and hair-like filamentous polymeric organelles (pili) to mediate attachment to host surfaces for colonization and biofilm formation [1–4]. These proteinaceous surface components often target extracellular matrix (ECM) components (e.g., collagen, fibrinogen, fibronectin, laminin, mucin, and their associated glycans). Similar to MSCRAMMs, the pilins (tip, backbone, and basal) or pilus subunits, which are building blocks of pili, contain signal sequence (SS) at the N-terminal and cell wall sorting signal (CWSS) at the C-terminal for their transportation across transmembrane and anchoring onto the cell wall, respectively.

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The CWSS includes a canonical sorting motif LPXTG (where X denotes any amino acid), a hydrophobic stretch region, and a positively charged tail essential for cell wall anchoring [5]. A transpeptidase enzyme called sortase catalyzes anchoring by recognizing the LPXTG motif. The sortase cleaves the bond between the threonine (Thr) and glycine (Gly) residues in the LPXTG motif and forms an acyl-enzyme intermediate. A nucleophilic attack by the amino group from the peptidoglycan precursor resolves the intermediate and creates an amide bond between the C-terminal of the threonine and the amino group of the peptidoglycan cross-bridge [6]. Similarly, sortase makes the covalent link between the pilins during the pilus assembly through the transpeptidase reaction [7]. Here, a nucleophilic attack by the amino group of a linking lysine from the N-terminal domain of adjacent pilin resolves the acyl-enzyme intermediate and forms an amide bond between threonine and lysine. The pilins (except tip pilins) often contain a pilin motif with a conserved linking lysine to facilitate sortase-mediated covalent links between the pilins. The genes for pilins (tip, shaft, and basal) and sortase (pilin-specific), which catalyzes pilus polymerization, are found together in the genome (pilus operon or island) for their synchronized expression. In contrast, the sortase (housekeeping), which catalyzes the cell wall anchoring, is located elsewhere in the genome [2].

X-ray crystallography has become one of the most favorable techniques to obtain three-dimensional structures of surface proteins, including the pilins and sortases, in understanding the mechanism of adhesion, pilus assembly, and targeting host-bacterial interaction for combating infections and health benefits [8–11]. Variants of immunoglobulin (Ig)-like domains, often with stabilizing isopeptide referred to as CnaA (DEv-Ig fold) and CnaB (Inv-Ig fold), are common building blocks in the surface protein and pilins [8, 9]. The Ig-like domains at the proximal end often form a stalk to project receptor-binding domains at the distal end of molecules or pili away from the cell surface to facilitate interaction with host surfaces. The structural knowledge of the surface molecules and their complexes with receptors is crucial in targeting host-bacterial interaction to improve health and combat infections [10]. X-ray crystallography is a successful and widely used technique for obtaining three-dimensional structures at the atomic level; however, a high amount of pure, soluble, stable, and homogeneous samples and their diffraction-quality crystals are prerequisites for the technique. With recent advancements and automation in the X-ray crystallography technique [12, 13], crystal structure can be solved in a few days or hours. However, there are still challenges in each step (Fig. 1), from protein production to structure determination; specifically, the crystallization step remains the bottleneck which limits the success rate [14]. A preliminary bioinformatic analysis in designing a recombinant construct,

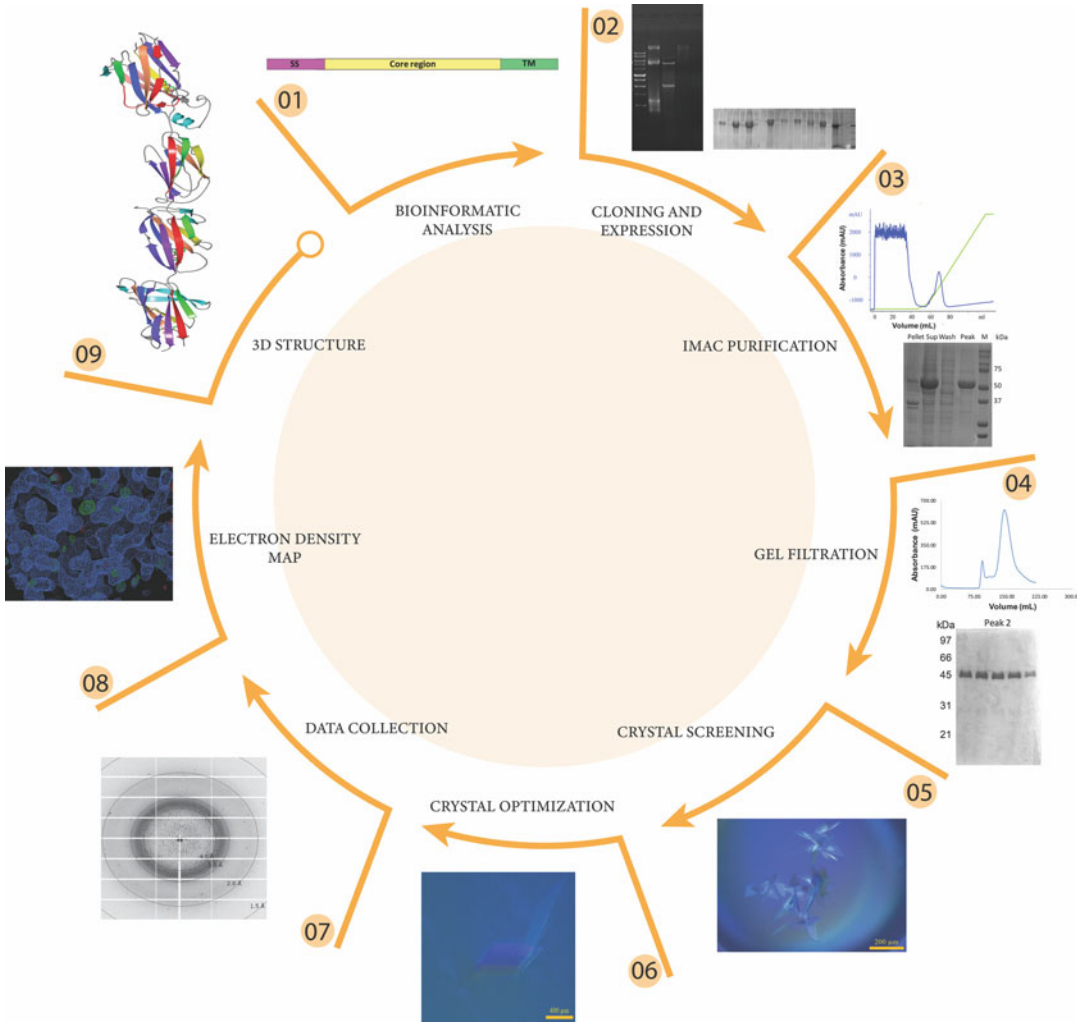

Fig. 1 An overview of protein structure determination by X-ray crystallography

optimal expression condition, efficient purification strategy, examination of samples by biophysical techniques before the crystallization step, extensive initial crystallization trials and subsequent optimization, and identifying suitable cryoprotectant and phasing techniques are crucial steps at the beginning of the method for avoiding frustrations and increasing the chances of success to obtain the structure in a short time.

We aim to provide a general protocol with tips for crystal structure determination of surface proteins and pilins anchored on the Gram-positive bacterial cell wall. The protocol involves recombinant protein production, crystallization, X-ray diffraction, data collection, and structure determination (Fig. 1). For theory and further details of the techniques, more focused reviews and complete manuals may be followed [15–19].

2 Materials

Prepare all the solutions with analytical-grade chemicals in fresh ultrapure and autoclaved water. Filter all solutions using a 0.22 μm filter to avoid contamination (*see* **Note 1**). Store the reagents, media, and solutions at the recommended temperature according to the manufacturer's instructions. Follow the appropriate disposal procedures while discarding waste reagents, media, and solutions after the experiments.

2.1 Bioinformatic Analysis of Protein Sequence

Use various bioinformatic tools to analyze protein sequences to identify the N-terminal signal sequence, a transmembrane region, a C-terminal cell wall sorting sequence (CWSS), secondary structures, disordered region, domain boundaries, etc., and calculate physical and chemical parameters. Some of the commonly used bioinformatics tools are listed below:

1. NCBI (https://www.ncbi.nlm.nih.gov/): provides access to biomedical and genomic information.
2. UniProtKB (https://www.uniprot.org/help/uniprotkb): provides comprehensive access to resources on protein sequence and functional information.
3. SignalP-6.0 (https://services.healthtech.dtu.dk/service.php?SignalP-6.0): predicts signal peptides.
4. TMHMM server 2.0 (https://services.healthtech.dtu.dk/service.php/TMHMM-2.0): predicts transmembrane regions.
5. CW-PRED (http://bioinformatics.biol.uoa.gr/CW-PRED/process.jsp): predicts the LPXTG and LPXTG-like cell-wall proteins of Gram-positive bacteria.
6. Translate tool-Expasy (https://web.expasy.org/translate/): allows the translation of a nucleotide (DNA/RNA) sequence to a protein sequence.
7. ProtParam server (https://web.expasy.org/protparam/): computes various physical and chemical parameters, including the molecular weight, theoretical pI, amino acid composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity.
8. InterPro (https://www.ebi.ac.uk/interpro/about/interpro/): predicts domains and functional sites.
9. Expasy (https://www.expasy.org/): provides access to various databases and software tools, including UniProtKB and ProtParam.
10. PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/): provides a range of protein structure prediction methods, including predictions for secondary structure, domains, disordered regions, homology models, etc.

11. SnapGene (https://www.snapgene.com): offers a fast and easy way to plan, visualize, and document molecular biology procedures.
12. OligoAnalyzer (https://www.idtdna.com/pages/tools/oligoanalyzer): identifies oligo properties, including melting temp, hairpins, dimers, and mismatches.

2.2 Gene Cloning

1. Disposable gloves.
2. Pipettes.
3. 0.22 μm syringe filter.
4. Lint-free wipes.
5. 1.5 mL microcentrifuge tubes (RNase-DNase free) and microcentrifuge tube stand.
6. A bacterial strain of interest (ATCC-American Type Culture Collection or other repositories of bacterial strains).
7. Genomic DNA Extraction Kit.
8. 10 μM forward and reverse primers (with restriction sites for enzyme inserted at 5′ end of each primer) for the gene encoding the protein with ectodomains or core structural and functional domains.
9. Pre-heated nuclease-free water at 65 °C for 10 min.
10. QIAprep Spin Miniprep kit.
11. Template DNA: genomic DNA/plasmid DNA.
12. Phusion high-fidelity DNA polymerase.
13. 10 mM dNTPs (deoxynucleotide triphosphates).
14. C1000 Touch thermal cycler (Bio-Rad).
15. Agarose gel electrophoresis assembly.
16. Agarose.
17. Tris-acetate-ethylene diamine tetra acetic acid (TAE, 1×) buffer: 40 mM Tris base, 20 mM acetic acid, 1 mM ethylene diamine tetraacetate (EDTA), pH 8.3.
18. Microwave.
19. Ethidium bromide (EtBr) 10 mg/mL.
20. Gel loading dye (6×): 10 mM Tris-HCl, pH 7.6, 0.03% bromophenol blue, 0.03% xylene cyanole FF, 60% glycerol.
21. 1 kb DNA ladder.
22. UV transilluminator.
23. UV imaging Gel Doc system.
24. QIAquick PCR Purification Kit.
25. Restriction enzymes and cut SMART buffer.
26. Surgical blades.

27. Water bath at 37 °C.
28. T4 DNA ligation buffer and T4 DNA ligase.
29. Competent cells (*E. coli* DH5α and BL21).
30. Luria-Bertani (LB) broth media.
31. UV spectrophotometer.
32. 0.1 M $CaCl_2$.
33. 15% Glycerol.
34. Liquid nitrogen.
35. LB agar.
36. Microcentrifuge.
37. Incubator shaker.
38. DNA sequencing facility.

2.3 Protein Expression

1. Sodium dodecyl-polyacrylamide gel electrophoresis (SDS-PAGE) assembly.
2. Resolving buffer: 1.5 M Tris-HCl, pH 8.8.
3. Stacking buffer: 0.5 M Tris-HCl, pH 6.8.
4. 30% acrylamide solution.
5. 10% sodium dodecyl sulfate (SDS) solution.
6. 10% Ammonium persulfate (APS) solution.
7. N, N, N, N′-Tetramethyl-ethylenediamine (TEMED).
8. Gel loading dye (4×): 40% glycerol, 200 mM Tris-HCl, pH 6.5, 8% SDS, 5% β-mercaptoethanol (β-ME), 0.04% bromophenol blue.
9. TGS buffer (10×): 250 mM Tris, pH 8.3, 192 mM glycine, 1% SDS.
10. Precision plus protein ladder.
11. Gel staining solution: 0.1% Coomassie brilliant blue, 40% methanol, 10% glacial acetic acid, 50% water.
12. Gel destaining solution: 40% methanol, 10% glacial acetic acid, and 50% water.
13. Transilluminator.

2.4 Large-scale Purification of Protein

1. Luria-Bertani (LB) broth media.
2. Antibiotic.
3. Isopropyl β-D-1-thiogalactopyranoside (IPTG) or another inducer for protein expression (*see* **Note 2**).
4. Incubator shaker.
5. Ultra-sonicator.
6. High-speed centrifuge.

7. Oakridge centrifuge tubes.
8. Lysis buffer: 50 mM phosphate buffer, pH 7.5 (**Note 3**), 400 mM NaCl supplemented with EDTA-free protease inhibitor cocktail.
9. Binding buffer: 50 mM phosphate buffer, pH 7.5, 400 mM NaCl, 20 mM imidazole.
10. Elution buffer: 50 mM phosphate buffer, pH 7.5, 400 mM NaCl, 400 mM imidazole.
11. HiTrap Chelating HP or HisTrap HP column (*see* **Note 4**).
12. Dialysis buffer: 20 mM Tris-HCl, pH 8.0, 150 mM NaCl.
13. Dialysis membrane (*see* **Note 5**).
14. Amicon Ultra centrifugal filters (*see* **Note 5**).
15. Tris buffer: 20 mM Tris, 150 mM NaCl, pH 8.0.
16. Gel-filtration columns such as HiPrep Sephacryl S200 HR (*see* **Note 6**).
17. An FPLC purification system.
18. Microcentrifuge.
19. Centrifuge.

2.5 Mass Spectrometry

1. Purified protein sample (~1 mg/mL).
2. Axygen microcentrifuge tubes (Merck, AXYMCT500C).
3. MS grade water.
4. 100 mM Ammonium bicarbonate.
5. 100% Acetonitrile.
6. MS grade trypsin.
7. Speed vacuum centrifuge.
8. 1% formic acid.
9. Sonicator water bath.
10. ZipTip pipette tips.
11. Mass spectrometer.
12. Data Analyst® software (SCIEX) and PeakView® 2.2 software (SCIEX).

2.6 Dynamic Light Scattering (DLS)

1. Purified protein sample (~2 mg/mL).
2. Tris-buffer: 20 mM Tris-HCl, pH 8.0, 150 mM NaCl.
3. Deionized water.
4. Quartz cuvette cell.
5. Zetasizer NanoZS90 (Malvern Panalytical) equipped with 4 mW He-Ne laser source ($\lambda = 633$ nm), and zetasizer software (Malvern Instruments Ltd).

2.7 Circular Dichroism (CD)

1. Protein sample in phosphate buffer (~0.2 mg/mL).
2. Phosphate buffer: 25 mM sodium phosphate, pH 8.0, 150 mM NaCl.
3. Deionized water.
4. Quartz cuvette cell ($d = 0.1$ cm).
5. CD spectrometer (Jasco Inc.).
6. Spectro-polarimeter (J-810, Jasco Inc.) with Spectra Manager™ Suite Spectroscopy Software (Jasco Inc.).

2.8 Initial Crystal Screening

1. Pre-crystallization test kit (HR2-142, Hampton Research).
2. Lab-made or commercial crystallization kits (**Note 7**).
3. Multi-channel pipette.
4. 96-well crystallization plates (HR3-082, Hampton Research) (*see* **Note 8**).
5. Crystal-clear sealing film (HR3-609, Hampton Research).
6. Automatic liquid handling systems such as Mosquito (SPT Lab Tech) (*see* **Note 8**).
7. Vibration-free cooled incubators (RUMED).
8. A stereo zoom microscope with camera options.
9. 24-well crystallization plates (VDX Plate HR3-141, Hampton Research) (*see* **Note 8**).
10. Siliconized coverslips (HR3-231, Hampton Research).
11. Grease sealant (HR3-508, Hampton Research).

2.9 Crystal Optimization

1. Stock solutions of salts, precipitants, and buffers with different pH values (*see* **Note 9**).
2. Additives (HR2-428, Hampton Research) (*see* **Note 9**).
3. Heavy-atom compounds (**Note 9**).
4. Stabilizing solution.
5. Seed bead kits (HR4-781, Hampton Research).
6. Proteases such as trypsin, α-chymotrypsin, subtilisin, etc., or Proti-Ace kit (HR2-429 and HR2-432, Hampton Research).
7. HEPES buffer: 50 mM HEPES, pH 7.5, 250 mM NaCl.
8. 1 M borane dimethylamine complex.
9. 1 M formaldehyde solution.
10. Tris buffer: 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT.

2.10 Cryocrystallography

1. Cryoloops with various diameters (ranging from 0.025 to 1 mm) (Hampton Research) (*see* **Note 10**).
2. Cryoprotectants (**Note 11**).
3. Liquid nitrogen.
4. Cryo gloves.
5. Protective face shield and goggles.
6. Crystal wand, forceps.
7. Cryogenic foam dewars of different sizes.
8. Micro-tool sets (Hampton Research).
9. Uni-pucks and accessories (Molecular Dimensions).
10. Crystal storage dewars.
11. Dry shippers (Molecular Dimensions).

2.11 Data Collection and Processing

1. Home source X-ray diffraction facility such as D8 Venture with photon detector and metal jet (Bruker) and Cryostream 700 (Oxford Cryosystems) (*see* **Note 12**).
2. Synchrotron sources such as European Synchrotron Radiation Facility (ESRF, France) (*see* **Note 13**).
3. Computer system (e.g., HP Z800 Workstations) with data storage and software tools for data processing and scaling such as HKL2000 [20], iMOSFLM [21], XDS [22], DUI/-DIALS [23], etc. (*see* **Note 14**).

2.12 Structure Solution, Model Building, and Deposition

1. Comprehensive software packages such as CCP4 (Collaborative Computer Project 4) package [24], Phenix [25], CNS (Crystallography and NMR system) [26], etc. (*see* **Note 15**).
2. Software for refinement and model building, such as REFMAC [27] and COOT [28].
3. Software tools for structural visualization and analysis such as PISA (https://www.ebi.ac.uk/pdbe/pisa/), CCP4MG [29], Chimera [30], and Pymol (https://pymol.org/2/).
4. Validation tools such as wwPDB validation tools (https://validate-rcsb-2.wwpdb.org/), MolProbity (http://molprobity.biochem.duke.edu/), Procheck (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/), etc.
5. PDB deposition server (https://deposit-pdbe.wwpdb.org/deposition).

3 Methods

Carry out all procedures at room temperature unless otherwise specified. The commonly used columns, chemical reagents, equipment, software tools, etc., for different techniques are mentioned in the method section for representation; however, these items from other manufacturers can be used in the following experiments.

3.1 Bioinformatic Analysis of Protein Sequence

1. Search in the NCBI or Uniprot database and download the gene sequence of the cell wall-anchored protein or pilin of interest from the respective bacterial strain.
2. Translate the downloaded gene sequence using the Translate tool or use translated protein sequence from UniProtKB if available.
3. Analyze the translated protein sequence of cell wall-anchored protein or pilin using SignalP, TMHMM, and CW-PRED servers to predict the N-terminal signal peptide, C-terminal sorting motif, and transmembrane regions (Fig. 2). Identify the core region to produce recombinant protein for crystallization. Use InterPro and PSIPRED for additional information, such as domain boundaries, secondary structure elements, and disordered regions (*see* **Note 16**).
4. Analyze the sequence of the core region using tools like ProtParam from expasy to predict molecular weight, theoretical pI, amino acid composition, extinction coefficient, estimated half-life, instability index, aliphatic index, and grand average of hydropathicity (GRAVY). Use the information about the core region in the cloning, purification, and crystallization, for example, in deciding the suitable pH of the buffer for protein.

3.2 Gene Cloning

1. Use the genomic DNA extraction kit to isolate the genomic DNA from the bacterial strain of interest obtained from ATCC or other sources.
2. Design primers (forward and reverse) specific to gene sequence (*see* **step 1** in Subheading 3.1) encoding the core region of the protein followed by the addition of suitable restriction sites.

Fig. 2 Schematic representation of N-terminal signal peptide (green), core region (yellow), C-terminal cell-wall sorting signal (CWSS) containing sorting region (orange, W) with LPXTG sorting motif (arrow), membrane-spanning hydrophobic region (grey, M), and cytoplasmic positively charged tail (blue, C) present in the sequence of cell wall-anchored protein or pilins

Use SnapGene and OligoAnalyzer tools if required in designing primers (*see* **Note 17**).

3. Synthesize the primers or obtain them from commercial services (Sigma-Aldrich).

3.2.1 Gene Amplification

1. Amplify desired DNA fragments by polymerase chain reaction. Thaw the PCR components on ice.
2. Prepare PCR reaction mix in a PCR tube by adding 1× Phusion HF buffer, 200 μM dNTPs, 0.5 μM forward primer, 0.5 μM reverse primer, 5–40 ng genomic DNA (*see* **step 1** in Subheading 3.2), 1-unit of Phusion DNA polymerase and make up the final volume to 50 μL with water (*see* **item 9** in Subheading 2.2).
3. Briefly centrifuge the mix (6000*g*, 1 min) and place it in a thermocycler (*see* **Note 18**).
4. Program thermocycler for the PCR reaction with the following cycling parameters; Initial denaturation (95 °C; 2–5 min); cycles of 25–35 with denaturation (95 °C; 30–50 s), annealing (55–65 °C; 45–60 s), and extension (72 °C; 30–60 s); and final extension (72 °C; 10–20 min).

3.2.2 Agarose Gel Electrophoresis

1. Dissolve 0.7–1 g of agarose in 100 mL of 1× Tris-acetate-ethylene diamine tetra acetic acid (TAE) buffer.
2. Boil the mix for 2–3 min at 99 °C. Cool the mix to a lukewarm temperature and add 2 μL ethidium bromide (EtBr).
3. Pour it into a horizontal gel-casting tray. Let it solidify.
4. Mix 2 μL of 6× gel loading dye with 10 μL of the PCR-amplified product.
5. Load and run the mix along with the DNA ladder on agarose gel at 70–90 V for 40–60 min.
6. Visualize the agarose gel using a UV imaging Gel Doc system. Bright white bands corresponding to the expected DNA size will appear on the gel.

3.2.3 Restriction Digestion

1. Use the PCR purification kit to isolate the remaining PCR product after the gel run from the rest of the PCR reaction mix.
2. Digest the purified PCR product and the recipient plasmid DNA (e.g., pET28a, which inserts the 6 X-His tag at the N- or C-terminal of protein is referred to herein for cloning, expression, and purification steps) (*see* **Note 19**) using the same set of restriction enzymes (*see* **step 2** in Subheading 3.2).
3. Prepare two separate digestion reaction mix for the PCR product and plasmid DNA. Mix 1 μg plasmid DNA/PCR product, 5 μL 10× cut SMART buffer, 1 unit each of both restriction

enzymes, and make the volume up to 50 μL with nuclease-free water (*see* **item 9** in Subheading 2.2).

4. Spin the mix for 1 min at 6000*g* and incubate at 37 °C for 4 h.
5. Run both the reaction mix on 0.7% agarose gel (*see* Subheading 3.2.2) along with the undigested PCR product, undigested plasmid DNA, and the DNA ladder.
6. Visualize the gel using a UV transilluminator. Orange fluorescent bands of DNA will be observed in the gel.
7. Excise the digested insert DNA and plasmid DNA bands from the gel using sterile surgical blades.
8. Extract the digested insert DNA and digested plasmid DNA from the gel using a QIAquick gel extraction kit.

3.2.4 DNA Ligation

1. Perform ligation of the extracted insert and plasmid DNA with the help of the T4 DNA ligase enzyme. Thaw the ligation components on ice.
2. Prepare reaction mix by adding reagents; 2 μL 10× T4 DNA ligase buffer, plasmid DNA (40–100 ng) and insert DNA in a 3:1 ratio, and 1-unit T4 DNA ligase, and make up the volume to 20 μL with nuclease-free water (*see* **item 9** in Subheading 2.2).
3. Briefly spin the mix (6000*g*, 1 min) and incubate overnight at 16 °C. Keep the mix at 4 °C until prepared for the transformation (*see* Subheading 3.2.6).

3.2.5 Competent Cell Preparation

1. Perform all steps in sterile conditions. Inoculate 2 mL of LB broth with a single colony of *E. coli* competent cells. Incubate the culture overnight at 37 °C while shaking at ~0.56 g.
2. Inoculate 100 mL of LB broth with 1% of overnight grown culture and allow it to grow at 37 °C with shaking at 0.56 g till OD_{600} (optical density at 600 nm) reaches ~0.5.
3. Rest the culture on ice for 15–30 min. Transfer the culture to sterile centrifuge tubes.
4. Pellet the cells by centrifugation at 2300*g* for 10 min at 4 °C. Discard the supernatant and resuspend the pellet in 10 mL ice-cold 0.1 M $CaCl_2$ and leave it on ice for 20–30 min.
5. Repeat **step 4** twice.
6. Finally, resuspend the pellet in 5 mL ice-cold 0.1 M $CaCl_2$ with 15% glycerol.
7. Make aliquots of 50 μL each with flash freezing in liquid nitrogen and store at −80 °C.

3.2.6 Transformation and Colony Confirmation

1. Transform the ligated product (*see* Subheading 3.2.4) into ultra-competent cells (*E. coli* DH5α) thawed on ice using the heat shock transformation method.
2. Mix 2–5 μL of the ligated product with competent cells, and allow to rest for 10 min on ice.
3. Give heat shock treatment to cells at 42 °C for 45–90 s, followed by 5–7 min of incubation on ice.
4. Add 850 μL of LB media to the cells and allow them to grow for 1 h at 37 °C with shaking at 0.56 g. Centrifuge the culture at 900*g* using microcentrifuge for 5 min.
5. Discard 800 μL of the supernatant and resuspend the pellet in the left-over media.
6. Plate 30 μL of the cells on LB agar plates with appropriate antibiotics and incubate overnight at 37 °C.
7. Confirm positive colonies from the plate using colony PCR, double digestion, and Sanger sequencing.
8. For colony PCR, mark 10–15 isolated colonies on the LB agar plate containing colonies. With the help of a pipette tip, touch the single colony and use it as a template in a PCR reaction mix (*see* **step 2** in Subheading 3.2.1).
9. Make a master plate of the colonies in parallel and keep it at 37 °C for 6–8 h.
10. Place the PCR reaction mix in a thermocycler (*see* **step 4** in Subheading 3.2.1).
11. Run the PCR samples on 1% agarose gel along with a DNA ladder and visualize the results using a UV imaging Gel Doc system. Positive colonies show bright white bands of the length of the amplified PCR product (*see* **step 6** in Subheading 3.2.2).
12. For double digestion, inoculate 5 mL LB broth supplemented with the appropriate antibiotic with the positive colony from the master plate. Incubate the culture overnight at 37 °C with shaking at 0.56 g.
13. Isolate the plasmid from the culture using a QIAprep Spin Miniprep kit.
14. Prepare a digestion reaction mix for the isolated plasmid with the same set of enzymes used for the digestion of PCR product and plasmids (*see* Subheading 3.2.3).
15. Run the digested plasmid on 1% agarose gel along with a non-digested plasmid and DNA ladder.
16. Visualize the agarose gel using a UV imaging Gel Doc system. Two white bands (upper band of plasmid and lower band of insert) will be visible for the plasmid-carrying insert.
17. Further, confirm the isolated plasmid with positive insert by Sanger sequencing.

Table 1
An experimental plan to screen conditions for optimal protein expression

IPTG Conc. (mM)	Incubation temp. (°C)	Incubation time (h)
0.1	37 25 18	3 12–16 12–16
0.5	37 25 18	3 12–16 12–16
1	37 25 18	3 12–16 12–16

3.3 Protein Expression

1. Transform 2 μL of the confirmed plasmid carrying the insert into an expression host, ultra-competent *E. coli* BL21 cells using the heat shock transformation method (*see* **Steps 1–6** in Subheading 3.2.6).
2. Pick a single colony of transformed *E. coli* BL21 cells from the plate and inoculate 5 mL of LB media containing the appropriate antibiotic and allow it to grow for 12–16 h at 37 °C in a shaking incubator at 0.56 g.
3. Prepare glycerol stock by adding 500 μL overnight grown culture to 500 μL of 50% glycerol. Freeze the glycerol stock at −80 °C.
4. Use 1% of overnight grown culture (*from* **step 2**) to inoculate 10 mL of LB media and grow at 37 °C in a shaking incubator with 0.56 g till OD_{600} reaches 0.6.
5. Optimize the protein expression by varying incubation temperatures (18–37 °C), incubation time (3–16 h), and concentration of inducer (0.1–1 mM of IPTG) (Table 1).
6. Compare the overexpression conditions along with negative control (non-induced culture) by analyzing the samples on SDS-PAGE.
7. Prepare a 12% resolving gel mix (*see* **Note 20**) by adding 1.3 mL of resolving buffer, 2.0 mL of 30% acrylamide solution, 1.5 mL of Milli Q autoclaved water, 0.1 mL of 10% SDS, 0.1 mL of 10% APS, and 0.02 mL of TEMED.
8. Pour the mix into the preassembled gel cassettes and allow it to form a gel. Overlay with water or isobutanol at the top to level the resolving gel solution and prevent contact with atmospheric oxygen. Allow the gel to polymerize.
9. Prepare a staking gel by adding 0.62 mL of stacking buffer, 0.33 mL of 30% acrylamide solution, 1.48 mL of Milli Q

autoclaved water, 0.02 mL of 10% SDS, 0.02 mL of 10% APS, and 0.005 mL of TEMED.

10. Pour the staking gel mix after removing overlayed water or isobutanol and insert a 10-well gel comb immediately without introducing air bubbles. Allow it to polymerize.
11. Dilute the 10× TGS buffer (*see* **item 9** in Subheading 2.3) by adding 100 mL buffer to 990 mL water to make 1× running buffer. Rinse the gel with water and transfer it into a container (Mini-PROTEAN Tetra cell system (Bio-Rad)) filled with 1× running buffer.
12. Mix 20 μL of the induced and uninduced sample with 6 μL of 4× gel loading dye (*see* **item 8** in Subheading 2.3). Heat the samples at 99 °C for 10 min. Spin the samples at 900 g using microcentrifuge for 1 min.
13. Load 10 μL of each sample into the wells along with the protein ladder. Close the container with a lid and run at 120–160 V until the dye front reaches the end of the gel.
14. Disassemble the gel plates carefully and transfer the gel into the staining solution (*see* **item 11** in Subheading 2.3) for 10–15 min. Wash the gel with water. Transfer gel to the destaining solution (*see* **item 12** in Subheading 2.3) until completely destained.
15. Visualize the gel under a transilluminator to identify the best expression conditions, which show a band for the protein of interest with expected molecular size and no equivalent band in uninduced samples.

3.4 Large-scale Purification of Protein

1. Using a sterile tip or loop, scrape the frozen bacteria from glycerol stock (*see* **step 3** in Subheading 3.3) and inoculate 10 mL LB media containing the appropriate antibiotic. Allow it to grow overnight at 37 °C in a shaking incubator at 0.56 g.
2. Add the overnight grown culture in 1 liter LB media supplemented with the appropriate antibiotic, and allow it to grow at 37 °C while shaking at 0.56 g until OD reaches 0.6.
3. Induce protein expression by adding optimal concentration of IPTG and grow the culture at the optimized condition for best protein expression (*see* **step 5** in Subheading 3.3).
4. Harvest the cells by centrifugation at ~10,000*g* for 20–30 min at 4 °C. Resuspend the pellet in 25 mL of lysis buffer (*see* **item 8** in Subheading 2.4).
5. Lyse the cells by sonication with 10–15 cycles of 10 s pulse on and 50 s pulse off in a glass beaker while keeping it on ice. Transfer the culture to oak ridge centrifuge tubes.
6. Centrifuge the lysed cells at 16,000*g* for 60 min at 4 °C, and filter the supernatant using a 0.22 μm syringe filter.

3.4.1 Immobilized Metal Affinity Chromatography (IMAC)

1. Equilibrate a 5 mL HisTrap column (*see* **Note 21**) with binding buffer (*see* **item 9** in Subheading 2.4) manually or through AKTA FPLC. Load the filtered supernatant onto the column and collect flow-through fractions.
2. Wash the column with the five-column volume of binding buffer.
3. Wash the column with a 5–10% gradient of elution buffer (*see* **item 10** in Subheading 2.4) to remove loosely bound proteins.
4. Collect the fractions during the wash with binding buffer and initial gradient (5–10%) of elution buffer (*see* **Note 22**).
5. Elute the bound protein using a linear gradient of elution buffer, and collect the eluted fractions.
6. Run the collected fractions in the SDS-PAGE gel (*see* **steps 7–15** in Subheading 3.3) to ensure the presence and purity of the recombinant protein.
7. Confirm the identity of the purified protein and its intact mass using mass spectrometric (peptide fingerprinting and intact mass) analysis if required (*see* **Note 23**).

3.4.2 Mass Spectrometry

1. Run the protein samples on 12–15% SDS-PAGE and prepare samples by either in-gel or in-solution digestion method using an appropriate MS grade trypsin protease as per the standard protocol.
2. For the in-gel digestion method, excise the single band from the gel using a sterile blade.
3. Completely destain the gel using a 50 mM ammonium bicarbonate (ABC) and 50% acetonitrile (ACN) solution.
4. Add 100% ACN for 5 min to the gel piece. Vacuum dry the fragments at 30 °C until completely dry. Follow the protocol for enzymatic digestion (*see* **step 6**).
5. For the in-solution digestion method, dilute the purified protein sample to a concentration of approximately 1 mg/mL in 25 mM ABC. Follow the protocol for enzymatic digestion (*see* **step 6**).
6. For enzymatic digestion (*see* **Note 24**), digest the vacuum-dried gel fragment/diluted protein sample overnight with MS grade trypsin gold (20 μg/mL) either solely or in combination with other proteases such as AspN endopeptidase at 37 °C in 25 mM ABC. Add 1% formic acid to stop the reaction.
7. Gently agitate the sample containing the microcentrifuge tube and sonicate it in a water bath for 20 min.
8. Centrifuge the microcentrifuge tube at 16,000*g* for 15–20 min.

9. Remove the supernatant-containing peptide in a fresh sterile microcentrifuge tube. Vacuum-dry the peptides at 30 °C for ~1 h. Rehydrate the sample in MS-grade water containing 0.1% formic acid (FA) and 2% ACN. Follow sample clean-up protocol (*see* **step 10**) using C-18 ZipTip pipette tips.
10. For sample preparation through ZipTip cleaning, equilibrate the tips with 100% ACN by aspirating and dispensing thrice. Repeat the equilibration step thrice with MS-grade water containing 0.1% formic acid. Bind the peptides to the tip by 10 cycles of aspirating and dispensing. Wash the tips with MS-grade water containing 0.1% formic acid by six times by aspirating and dispensing. Elute the peptides in 70% ACN containing 0.1% formic acid. Vacuum dry the peptides at 30 °C until completely dry.
11. Resuspend the dried sample in 20 μL of MS-grade water containing 2% ACN and 0.1% formic acid. Use the sample for mass spectrometry analysis.
12. For intact mass spectrometry sample preparation, dilute the protein sample to ~1 μg/mL in the buffer in which it is purified. Spin the sample at 16,000*g* using microcentrifuge for 40–50 min. Use the sample for mass spectrometry analysis.

3.4.3 Size Exclusion Chromatography

1. Pool the fractions containing the protein of interest after affinity chromatography and perform dialysis overnight against dialysis buffer (*see* **item 12** in Subheading 2.4) at 4 °C using a membrane of appropriate molecular weight cut-off (*see* **item 13** in Subheading 2.4).
2. Concentrate the protein using an Amicon centrifugal filter (equilibrated with the binding buffer) of appropriate molecular weight cut-off (*see* **Note 25**).
3. Equilibrate the size exclusion column with more than one column volume of size-exclusion buffer (*see* **item 15** in Subheading 2.4) (*see* **Note 21**).
4. Load the concentrated protein (~1 mL) onto a size exclusion column, and monitor the elution of protein at wavelength UV280 nm while collecting fractions.
5. Analyze the fraction by SDS-PAGE (*see* **steps 7–15** in Subheading 3.3) and pool the fractions containing pure protein.
6. Concentrate the pooled sample to a desired concentration using Amicon centrifugal device (*see* **item 14** in Subheading 2.4) (equilibrated with Tris buffer) for crystallization trials (*see* **Note 25**).

3.5 Initial Crystal Screening

1. Confirm the homogeneity of the protein sample by looking at the chromatographic profile of size-exclusion chromatography and/or using DLS (*see* **Note 26**).
2. Check the proper folding and presence of secondary structural elements of purified protein by biophysical techniques such as CD (*see* **Note 27**).
3. Start initial crystallization trials with a pure and homogeneous protein at a concentration of 5–20 mg/mL in low-concentration buffer (e.g., 20 mM Tris with low or no salt). Alternatively, identify the suitable buffer and optimal concentration of protein by screening samples at various buffer conditions with DLS or other biophysical techniques and performing pre-crystallization tests (*see* **Note 28**), respectively.
4. Fill each well of the sitting-drop crystallization plate (96-well) with 60 μL reagent or mother liquor from commercial screens (*see* **item 2** in Subheading 2.8) (*see* **Note 8**). Use a multi-channel pipette if required to fill wells.
5. Set up the sitting drop with a volume of ~200 nL on each well by mixing protein and mother liquor in a 1:1 ratio using an automated liquid handling system (*see* **item 6** in Subheading 2.8).
6. Cover the crystallization plate with clear film (*see* **item 5** in Subheading 2.8), examine the drops in the plate under a microscope, and then place it in a vibration-free incubator with the desired temperature (*see* **Notes 29** and **30**).

3.6 Crystal Optimization

1. Examine the plates under a stereo-zoom microscope for initial hits.
2. Use the crystal for the X-ray diffraction experiment if the initial hits are suitable single crystals; else, reproduce them for screening cryoprotectants (*see* **item 2** in Subheading 2.10) or preparing heavy atom derivatives (*see* **item 3** in Subheading 2.9).
3. Optimize the condition if the initial hits are microcrystals, needles, clusters, small plates, and spherulites.
4. Use a hanging-drop vapor-diffusion method with a 24-well plate for reproduction or optimization.
5. Apply sealant (*see* **item 11** in Subheading 2.8) on each well or use sealant-coated plate for plate setup. Fill the wells with ~0.7–1 mL of mother liquor, which gave initial hits for reproduction. Add 1 μL of protein onto a clean siliconized cover slip, add 1 μL of mother liquor, and gently mix by dispensing and withdrawing with a pipette and avoiding air bubbles. Invert the coverslip, place it on the well and seal it.
6. Use the same procedure (*see* **step 5**) for optimization with a 24-well plate but well filled with mother liquor according to the experimental design of optimization. Use the condition of

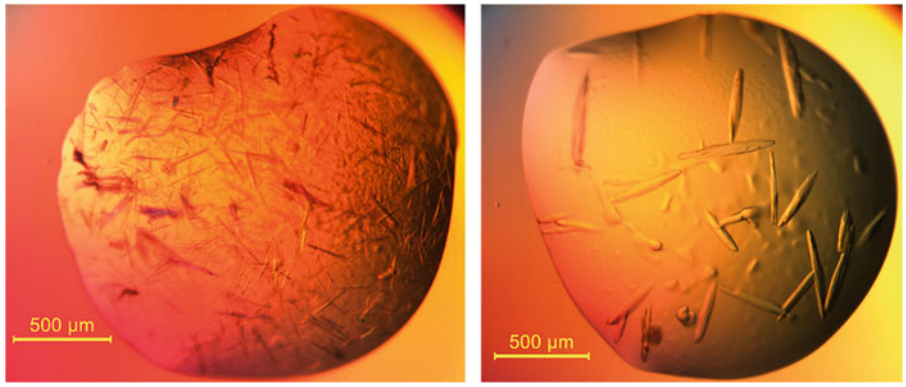

Fig. 3 Crystallization drops of a pilus protein. A drop with thin needle-like crystals before optimization (left). A drop with thick rod-like crystals after the optimization step (right)

Table 2
An experimental plan for crystal optimization in a 24-well crystallization plate

	1	2	3	4	5	6
A	20 mM HEPES pH 6.8 + 15% PEG 6000	20 mM HEPES pH 7.0 + 15% PEG 6000	20 mM HEPES pH 7.2 + 15% PEG 6000	20 mM HEPES pH 7.4 + 15% PEG 6000	20 mM HEPES pH 7.6 + 15% PEG 6000	20 mM HEPES pH 7.8 + 15% PEG 6000
B	20 mM HEPES pH 6.8 + 20% PEG 6000	20 mM HEPES pH 7.0 + 20% PEG 6000	20 mM HEPES pH 7.2 + 20% PEG 6000	20 mM HEPES pH 7.4 + 20% PEG 6000	20 mM HEPES pH 7.6 + 20% PEG 6000	20 mM HEPES pH 7.8 + 20% PEG 6000
C	20 mM HEPES pH 6.8 + 25% PEG 6000	20 mM HEPES pH 7.0 + 25% PEG 6000	20 mM HEPES pH 7.2 + 25% PEG 6000	20 mM HEPES pH 7.4 + 25% PEG 6000	20 mM HEPES pH 7.6 + 25% PEG 6000	20 mM HEPES pH 7.8 + 25% PEG 6000
D	20 mM HEPES pH 6.8 + 30% PEG 6000	20 mM HEPES pH 7.0 + 30% PEG 6000	20 mM HEPES pH 7.2 + 30% PEG 6000	20 mM HEPES pH 7.4 + 30% PEG 6000	20 mM HEPES pH 7.6 + 30% PEG 6000	20 mM HEPES pH 7.8 + 30% PEG 6000

the initial hit to prepare an experimental plan for optimization. Vary parameters, such as the pH of the buffer, the concentration of salt and precipitants, etc., during optimization. For example, if the initial hit is obtained in 20 mM HEPES, pH 7.5 and 25% PEG 6000, vary buffer pH (6.8, 7.0, 7.2, 7.4, 7.6, and 7.8) in one direction and concentration of PEG (15, 20, 25, 30) another direction in a 24-well plate for screening (Fig. 3) (e.g., Table 2) (*see* **Note 31**).

7. Use micro or macro seeding techniques if reproduction and optimization fail to produce crystals. Transfer crystals from the initial condition to new suitable drops by viewing them under the light microscope to grow them (macro seeding).

8. Alternatively, crush the crystal or crystalline-like materials in a stabilizing solution (i.e., mother liquor with slightly higher precipitant concentration, in which crystal does not dissolve) from the initial hit with a glass tissue homogenizer or seed bead (*see* **item 5** in Subheading 2.9) to make seed stock, then serially dilute and use it to seed a new drop using a pipette or animal whisker (micro seeding) (*see* **Note 32**).
9. Screen additives (*see* **item 2** in Subheading 2.9) and in situ proteolysis (*see* **item 6** in Subheading 2.9) by adding them in the drops during optimization to obtain better crystals or improve diffraction quality if required. Producing stable fragments by limited proteolysis for crystallization or improving diffraction quality has been successfully used for different pilins [31–34].

3.6.1 Lysine Methylation

1. Optionally, perform surface lysine methylation [35, 36] to improve the diffraction quality of crystals or to obtain crystals. Dialyze the purified protein fraction (~1 mg/mL) overnight at 4 °C with HEPES buffer.
2. Add 200 μL of freshly prepared 1 M borane dimethylamine complex and 400 μL of 1 M formaldehyde solution to the dialyzed protein.
3. Place the mix in shaking at 4 °C for 2 h.
4. Repeat **steps 2** and **3** once.
5. Add 100 μL of 1 M borane-dimethylamine complex and incubate overnight with gentle shaking at 4 °C.
6. Centrifuge the protein solution at 20,000*g* for 40–50 min at 4 °C.
7. Concentrate the supernatant (~1 mL) in an Amicon centrifugal filter.
8. Load the concentrated fraction onto a size exclusion column pre-equilibrated with Tris buffer (*see* Subheading 3.4.3). Concentrate the fractions containing protein and perform crystallization or optimization trials.

3.7 Cryo-crystallography

1. Perform a cryo screening to identify a suitable cryoprotectant to avoid radiation damage to the crystal and increase diffraction quality.
2. For cryo-screening, prepare cryoprotectant solution by mixing a cryoprotectant (*see* **item 2** in Subheading 2.10) and mother liquor or stabilizing solution in a ratio of 2:8 or 3:7.
3. Transfer the crystal from the drop to the cryoprotectant solution using a nylon loop under the microscope.

4. Pick up the crystal immediately from the cryoprotectant solution and flash freeze in liquid nitrogen for storing or mount it on the goniometer in the path of the cryo stream for data collection (*see* **Note 33**).

3.8 Data Collection and Processing

1. Mount the crystal on the goniometer using a loop of appropriate size and center the crystal on the beam either manually or using an automated procedure with computer vision assistance.
2. Collect a few reference frames (e.g., at 0° and 90°) to examine the X-ray diffraction pattern, centering, and indexability to determine the unit-cell dimensions and space group (*see* **Note 34**).
3. Use the information from this preliminary analysis to set up a strategy for X-ray diffraction data (*see* **Note 35**).
4. Use the control software (graphical user interface tool) in the computer connected to the X-ray diffraction system (e.g., PROTEUM3 for D8 VENTURE from Bruker Metal Jet and MXCuBE at ESRF beamlines) to feed optimal parameters (e.g., exposure time, image width, rotation range, detector distance, beam size, and wavelength) as per strategy for collecting X-ray diffraction data.
5. Process the collected data with data processing software tools (HKL2000, XDS, MOSFLM, DIALS, etc.) through the steps of spot finding, indexing (assigning the correct Miller indices h, k, l index to each spot on a diffraction image), integrating (measuring spot intensities for each spot in diffraction images), scaling and merging (scaling and merging the reflections from the collected images identified by the indices h, k, l) (*see* **Notes 36** and **37**). The final reflection (.mtz) file, after the data processing and scaling steps, typically contains Miller indices of reflection, averaged intensity, and standard deviation (H, K, L, I, and σI). This X-ray diffraction data collected from native crystal is generally referred to as native data.
6. Look at the data statistics after initial processing and reprocess the data if required. Aim to get best statistics, i.e., a maximum completeness (~99%), best I/σI (i.e., signal-to-noise ratio, I – the intensity of the signal and σ – standard deviation), multiplicity, and resolution, and low merging R values (R_{sym} or $R_{merge} < 10\%$, i.e., disagreement index between symmetry-related reflections) (*see* **Note 37**).
7. Additionally, perform an anisotropic correction using the STARANISO server if the X-ray diffraction shows the anisotropic property.

3.9 Structure Solution and Refinement

1. Calculate phase information, which is lost during X-ray diffraction experiments and required for structure determination, using theoretical or experimental phasing approaches.
2. Use the theoretical phasing method (i.e., molecular replacement (MR)) as the first choice if a suitable model template is available in the PDB from homologous protein (>30% sequence identity) or suitable model from structure prediction methods (homology modeling, threading, and ab initio techniques). In the absence of homologous templates due to the low sequence identity (<28%), a fragment-based ensemble approach may also be used to generate a model template from distant structural homologs [31, 37]. Software tools such as MOLREP, PHASER, and AMORE from the CCP4 suite can be used for MR, which find the rotation and translation that position the model in the unit cell to give the highest correlation between experimental diffraction measurements and those calculated from the model (*see* **Note 38**). Use Matthews coefficient calculation to estimate the number of molecules in the asymmetric unit for MR.
3. Check if a unique solution with a strong signal is found at the end of MR (*see* **Note 39**), or else attempt experimental phasing methods (*see* **step 4**).
4. Use one of the experimental phasing methods (single-wavelength anomalous dispersion (SAD), single-wavelength isomorphous dispersion (SIR), SIR with anomalous scattering (SIRAS), multiple-wavelength isomorphous replacement (MIR), or MIR with anomalous scattering (MIRAS)) which requires the presence of either intrinsic metals (e.g., Zn, Fe, S, or incorporated Se (*see* **step 5**)) or introduced heavy atoms (Au, Pt, Pb, Au, Os, Ir, Yb, Tb, I, Br, etc.) in the protein crystal.
5. Prepare selenium-incorporated protein and crystal if there are enough methionine residues in the protein for Se-SAD or Se-MAD phasing for experimental phasing [38–41] (*see* **Note 40**). Collect anomalous data at the wavelength of the peak (absorption edge) to obtain the maximum anomalous signal for Se-SAD phasing. Additionally, collect data at an inflection point and high-energy remote if required for Se-MAD phasing. The presence of heavy atoms and anomalous signals can be examined through fluorescence scan at Synchrotron sources or while processing and checking the data (*see* **Note 41**). If the anomalous signal is insufficient, attempt to collect anomalous data using a similar strategy for endogenous metal if present or identified in the fluorescence scan at Synchrotron sources, or else go for heavy atom derivatization (*see* **step 6**).

6. Prepare heavy atom derivatives either using a halide quick-soaking method (soaking of protein crystals in a cryo-solution containing halides such as bromide or iodide anions for less than 1 min) [33, 34, 42] or classical methods of heavy-atom derivatization [43, 44], which involves soaking of protein crystals in a stabilizing solution or mother liquor containing a heavy atom compound. This trial-and-error method requires screening multiple heavy atom compounds to find an optimal concentration of heavy atom compound and soaking time. The success of the method depends on several factors (e.g., the binding capacity of heavy atom compound, survival of crystal during the soaking, diffraction quality of the soaked crystal, the strength of an anomalous signal, etc.). Alternatively, co-crystallization can be attempted by mixing heavy atom solution with protein and incubating before crystallization trials. Additionally, lanthanide complexes can also be explored to prepare heavy atom derivatives to obtain high anomalous signal at home sources or synchrotron sources [45, 46].
7. Collect diffraction data from heavy atom derivative crystals at the peak wavelength of a respective heavy atom or at the reachable point which gives the maximum signal, and check for the presence of signal using a similar strategy mentioned for Se-SAD phasing (*see* **step 5**). The presence of heavy atoms and anomalous signals can also be examined through fluorescence scan at Synchrotron sources and also to decide the wavelength for data collection.
8. Use one of the experimental phasing programs, such as CRANK and autoSHARP from the CCP4 suite, to identify heavy atom sites and obtain phases to calculate the initial electron density map (*see* **Note 42**). The scattering factors (f′ and f′′) required in experimental phasing programs can be determined experimentally by carrying out a fluorescence scan at Synchrotron sources or estimated theoretically for a given wavelength and anomalous scatterer from the tables of anomalous scattering coefficients or using a server such as X-ray Anomalous Scattering at Biomolecular Structure Center (BMSC) at University of Washington (http://skuld.bmsc.washington.edu/scatter/AS_periodic.html).
9. Use the initial electron density map calculated by one of the phasing methods for further improvement using density modification to obtain an interpretable electron density map. The density modification improves the phases by selecting the correct phase from two possibilities in experimental phasing or reducing bias toward the search model in MR. Phase improvement is performed using solvent flattening, histogram matching, and optionally non-crystallographic symmetry averaging (*see* **Note 43**).

3.10 Model Building and Refinement

1. Use the electron density map for automated model building with tools such as ARP/WARP and BUCCANEER in the CCP4 to generate the initial model.
2. Use COOT and REFMAC from CCP4 or PHENIX for further manual model building and refinements, i.e., the process of improving agreement between the structural model and the experimental data (structure factors and electron density maps).
3. Repeat the cycles of model building and refinement, and monitor crystallographic values of R_{work} and R_{free} (a measure of the disagreement between the observed amplitudes (F_o) and the amplitudes calculated from the model (F_c)), which should decrease gradually as the quality of the model gets better and better over the cycles.
4. Examine the electron density map for metal ions or any modifications such as isopeptide bonds, disulfide bridges, etc., and include them. Optionally, TLS (Translation-Libration-Screw-rotation), restraints, and anisotropic refinement can be carried out based on the structural features of proteins and data resolution. Additionally, difference map, omit map, and anomalous map can be used to check the presence of metals, ligands, and other critical parts of the molecule.
5. Add water molecules and perform the final cycles of refinement.
6. Stop the refinement cycles when there is no further improvement in the model quality or a decrease in R_{work} and R_{free}.

3.11 Validation and Deposition

1. Use validation tools (Ramachandran map, unmodeled blobs, difference map peaks, check/delete waters, analysis of geometry, temperature factor, rotamer, clashes, and density fit) in the COOT for initial validation during the final cycles of refinement to correct the outliers in the Ramachandran map and other model errors (*see* **Note 44**).
2. Submit the coordinates and structure factors wwPDB validation system (https://validate-rcsb-1.wwpdb.org) for final validation. Carefully go through the validation report and correct the indicated errors in the report.
3. Fill in the related information such as details of protein, crystal, X-ray data collection, processing, structure solution, and refinement.
4. Review the filled information and submit it. A PDB ID will be issued after the acceptance (*see* **Note 45**).

4 Notes

1. Always wear gloves during cloning and protein purification to avoid contaminating the sample with any DNase and keratin.
2. The type of inducer used for protein induction depends on the vector carrying the gene for the protein. The most commonly used vectors (pET28a, pET28b, and pET23a) require IPTG as the inducer.
3. Check the pI of the protein using ProtParam to decide the buffer and pH. Keep buffer at least 1.0-unit pH away from the pI of protein (e.g., if protein pI is 6.0, then the pH of buffer used for protein purification is either 5.0 or 7.0).
4. Use HiTrap Chelating HP or HisTrap HP column for histidine-tagged protein purification and GSTrap column (17513101, GE Healthcare) for GST-tagged protein purification.
5. The dialysis membrane and the Amicon centrifugal filters are available in different molecular weight cut-offs (3 kDa, 10 kDa, 30 kDa, etc.). The choice of cut-off will depend on the molecular weight of the protein. Choose the membrane with a molecular weight cut-off that is 2–3 times smaller than the molecular weight of the protein.
6. The size exclusion column should be chosen based on the molecular weight of the protein. For example, HiLoad 16/600 Superdex 75 pg, 28989334; HiPrep Sephacryl S100 HR, 17119401, HiPrep 26/60 Sephacryl S-500 HR, 28935607 from GE Healthcare. Additional chromatographic methods, such as ion exchange chromatography, may also be used if the protein is not pure after purification by the two steps (IMAC and SEC).
7. The most commonly used commercial screens are Crystal Screen (Hampton Research), PEGs Suite (NeXtal), PACT Suite (NeXtal), Top96 Crystallization Screen (Anatrace), PACT Premier (Molecular Dimensions), etc. A complete list of available crystallization kits can be found on the manufacturer's website.
8. The most commonly used 96-well and 24-well crystallization plate is listed for representation; however, various plate models with different sizes and wells are commercially available for sitting and hanging drop, and micro-batch methods. Similarly, various automatic liquid handling systems are available from different manufacturers (e.g., Crystal Phoenix (Art Robbins Instruments, LLC), and HoneyBee (Digilab Genomic Solutions)).

9. Stock solution of various salts (ammonium sulfate, sodium chloride, magnesium chloride, etc.), precipitants (polyethylene glycol, propanol, hexanediol, etc.), and buffers with different pH values (MES, HEPES, PBS, Tris, etc.) can be prepared manually for optimization screens. Various additives (multivalent metal ions, salts, amino acids, polyamines, reducing agents, detergents, polymers, chelating agents, carbohydrates, and volatile and non-volatile organic compounds (e.g., HR2-428, Hampton Research) can also be used in the optimization trials. For heavy atom derivatization, various heavy atom compounds (Potassium tetra chloroplatinate (II), Silver nitrate, Mercury (II) acetate, Trimethyl lead acetate, etc.) or heavy atom screens (HR2-448 and HR2-450, Hampton Research) are available from different manufactures. Specialized heavy atom compounds for phasing are also commercially available, for example, I3C (Hampton Research), Tantalum and tungsten clusters (Jena bioscience), and lanthanide complex such as TbXo4 (Polyvalan Crystallophore, No.1, Molecular Dimensions) [32, 46].
10. Specialized mesh-type LithoLoops are also available in various sizes (Molecular Dimensions). Cryo-loops (Molecular Dimensions/Hampton Research), crystal caps, and types should be checked for compatibility with the Goniometer of the X-ray diffraction facility of home or synchrotron sources.
11. Some commonly used cryoprotectants are glycerol, PEG 400, ethylene glycol, 2-methyl-2,4-pentanediol, lithium acetate dihydrate, magnesium acetate tetrahydrate, etc. Commercial cryoprotectant screens are also available (CryoPro (HR2-074), Hampton Research; CryoProtX (MD1-61) and CryoSol (MD1-90), Molecular Dimensions).
12. Other diffraction systems from marXperts and Rigaku with different X-ray sources, detectors, and cryptosystems are also available for home sources.
13. Various synchrotron radiation facilities are available, for example, Advanced Photon Source (APS, USA), the ALBA synchrotron (Spain), Swiss Light Source (SLS, Switzerland), SPring-8 (Japan), Deutsches Elektronen Synchrotron (DESY, Germany), Elettra Synchrotron (Italy) and Raja Ramanna Centre for Advanced Technology (RRCAT, India).
14. A set of tools and programs are also available as a single package with a multi-processor version for automatic data processing – for example, autoPROC (Global Phasing Limited) [47].
15. The listed comprehensive packages contain modules for structure solutions by MR, SAD, MAD, SIR, MIR, SIRAS, MIRAS, and their combination.

16. Additionally, remove disordered regions if present either at the N- or C-terminal end to increase the chances of getting soluble, homogeneous protein and crystals.
17. Use the bioinformatic tools to check the properties of the primers, such as length (18–30 bases), GC content of primers (should be between 35–70%), hairpin loop, and dimer formation at high temperatures (>30°), and melting temperatures (Tm) (between 65 °C and 75 °C, and within 5 °C of each other).
18. If required, prepare positive and negative control reactions with and without templates of known size and appropriate primers, respectively, for confirmation.
19. The choice of vector depends on multiple factors (the type of purification method used, solubility, and size of the protein). For example, the pET28a vector can insert 6 X his tag at both N and C terminal or only at N-terminal, pET23a inserts a C-terminal 6 X his tag, and pGEX-6p adds a GST tag to the protein at N-terminal and also enhances the solubility of the protein.
20. The percentage of SDS PAGE gel depends on the size of the protein. For protein size >100 kDa, 10% gel is suitable. For protein size <30 kDa, 15% gel is recommended. The gel percentage can be varied by changing the volume of acrylamide and the volume of Milli Q water while the rest of the components of the resolving gel remain the same.
21. Refer to column manuals for optimal sample volume, flow rate, pressure, compatibility of buffer components, etc.
22. Fractions should be collected during sample loading, initial wash with binding buffer, and wash with 5–10% gradient of elution buffer. Analyzing these fractions on SDS-PAGE helps to examine if the protein of interest eluted during the wash if not found in the eluted fractions.
23. While preparing samples for Mass spectrometry, wear gloves, never touch the sample with your bare hand, and always tie your hair or cover it with a surgical cap to protect the sample from keratin contamination.
24. Before enzymatic digestion, treat both in-solution and in-gel samples with freshly prepared 10 mM dithiothreitol (DTT) in 100 mM ammonium bicarbonate overnight at room temperature to reduce the disulfide bonds for protein-containing disulfide bonds. Next, incubate with freshly prepared 55 mM iodoacetamide in 100 mM ammonium bicarbonate for 45 min at room temperature in the dark to alkylate cysteine residues.

25. Check the protein every 20–30 min while concentrating, and gently mix the protein in Amicon centrifugal filter using a pipette to avoid precipitation.
26. Look at the elution volume of the size-exclusion profile or DLS analysis to identify the oligomeric state (monomer, dimer, etc.) of the protein. Pool fractions from each peak of size-exclusion separately if there is more than one oligomeric state, and avoid pooling fractions containing aggregates. The protocol for performing DLS is as follows, filter the protein sample in Tris buffer, deionized water, and Tris buffer through 0.22 μm membrane filter. Centrifuge all the components at 16,000*g* using microcentrifuge for 1 h at 4 °C. Rinse the quartz cuvette cell with deionized water. Equilibrate the cuvette thrice using Tris buffer. Fill 20 μL protein sample in the cuvette. Gently wipe the outer surfaces of the cuvette with lint-free wipes. Place the cuvette into the instrument and record the profile.
27. The protocol for sample preparation for Circular Dichroism (CD) spectroscopy: dialyze the purified protein (~0.2 mg/mL) (obtained after size exclusion chromatography step) overnight at 4 °C against phosphate buffer: 25 mM sodium phosphate, pH 8.0, 150 mM NaCl. Filter the dialyzed protein sample, deionized water, and phosphate buffer using a 0.22 μm membrane filter. Centrifuge all the components at 20,000*g* for 1 h at 4 °C. Rinse the quartz cuvette cell using deionized water. Equilibrate the cuvette with phosphate-buffer. Fill 20 μL protein sample in the cuvette. Gently wipe the outer surfaces of the cuvette with lint-free wipes. Place the cuvette into the instrument, and record and analyze the spectrum.
28. Perform a pre-crystallization test to determine the optimal protein concentration for crystallization screening. In a 24-well crystallization plate, fill two wells separately with 1 mL of reagents A and B (provided in the pre-crystallization tests kit). On a glass cover slide, place 1 μL drop of protein and add 1 μL of reagent A. Mix the protein and reagent gently using a pipette. Invert the cover slide over the well containing reagent A and seal. Repeat the procedure for reagent B. Observe the drops under a stereo zoom light microscope after 30 min. Protein concentration is optimal for crystallization trials if the drops are with microcrystalline or light granular precipitate. Increase or decrease protein concentration if the drops are clean or amorphous precipitate, respectively.
29. Set up crystallization trials at different temperatures (e.g., 4 °C, 22 °C) in parallel if there is enough protein to increase the chances of getting crystals and screening.
30. Check the drops in the plate immediately after setup, once a day for a week and once a week or month, until finding crystal hits or drops dry.

31. Set up the second round of optimization based on the results from the first round of optimization with the appropriate grid size. The protein and mother liquor ratio can also be varied (e.g., 1:2 and 2:1) during the optimization instead of 1:1.
32. An automatic liquid handling system (robot) can also be used to dispense the seeds into the drops for large-scale screening.
33. Store the crystal in pucks or canes in the liquid nitrogen container to ship to the synchrotron for data collection or to preserve them for later experiments.
34. X-ray diffraction pattern from protein crystals exhibits many closely spaced diffraction spots. In contrast, crystals of salt or small molecules with small unit cells show a few reflections, which are stronger and far apart. Alternatively, other less direct approaches such as dehydration, crushing, dye absorption (e.g., Izit), a control drop setup (without a protein), SDS-PAGE analysis, and examination under a UV microscope can be used to distinguish protein crystals from salt crystals, before crystal optimization and initial X-ray diffraction steps.
35. The graphical interface tools at synchrotron sources will also give additional options for parameters such as X-ray energy or wavelength and transmission, and advanced options such as standard data collection, characterization, energy scan, etc. These options will help screen for anomalous scatters and change the wavelength in collecting anomalous data.
36. Most data processing software tools have options for automatic peak searching, indexing, lattice identification, integration, and scaling. Users can monitor the steps and interrupt if required when automatic steps fail or for manual processing.
37. Though the mean ($I/\sigma I$) along with R_{sym} and R_{merge} were traditionally used in deciding the cut-off for the resolution of data, R_{meas} or R_{pim}, and half-dataset correlation coefficient $CC_{1/2}$ (>0.30) are also commonly used in recent days [48] and these recommended criteria are also implemented in the automatic processing tools such as autoPROC [47].
38. Choose the resolution manually or let the program decide automatically. Carefully check the model bias (i.e., it tends to look more like the search fragment than it should).
39. For MR search, a unique solution with a strong signal can be identified by looking at log files or results from MR programs. For example, in Phaser MR, the Z-score (number of standard deviations above the mean value) can be checked for the correct solution, which will generally have a Z-score of translation function (TFZ) over 5 and be well separated from the rest of the solutions. Similarly, an LLG (log-likelihood gain) of 40 or greater usually indicates a correct solution.

40. Approximately a single selenium site per 100 residues is enough to get a sufficient anomalous signal to solve the structure [38]. Selenium-incorporation protocol: transform the recombinant plasmid carrying the protein of interest into ultra-competent *E. coli* B834 (DE3) cells (*see* Subheading 3.2.5), auxotrophic for methionine (Met) (*see* **steps 1–6** in Subheading 3.2.6). Inoculate a single colony observed after transformation from the LB agar plate in 10 mL LB broth supplemented with a respective antibiotic. Grow the culture overnight at 37 °C with 0.56 g. Centrifuge the culture at 6000*g* for 5 min at 4 °C. Wash the cell pellet with 5 mL of Sel-Met medium base (Molecular Dimensions) that contains no methionine. Repeat the centrifugation and washing steps twice. Resuspend pellet after the last wash in 5 mL of Sel-Met medium base and use as inoculum for 1 liter of Sel-Met medium base supplemented with Sel-Met medium nutrient mix (Molecular Dimensions) and 4 mL L-Met (Molecular Dimensions) as per the manufacturer's protocol and incubate at 37 °C with 0.56 g till OD_{600} reaches to 0.6. Induce the culture with 1 mM IPTG and grow at the optimized condition for best expression. Harvest cells by centrifugation at 9000*g* for 30 min and carry out purification by IMAC and size-exclusion chromatography (*see* Subheadings 3.4.1 and 3.4.3).
41. Procedures for checking anomalous signals may be found in the documentation of individual data processing programs. For example, indications of strong anomalous signals in the data can be reviewed in XDS by looking at the values of the *Anomal Corr* (>30%) or *SigAno* (>1). Similarly, in AIMLESS or SCALA of CCP4, values of anomalous correlation coefficient (ACC) and $\Delta F/\sigma(\Delta F)$ can be used.
42. Heavy atom sites with high occupancy (0.3–1) can be used in the initial round of phase calculation. After obtaining interpretable electron density (FOM > 0.3), weak sites may be included in later cycles of phase calculations.
43. Combination molecular replacement and experimental phasing are common if a search model and some experimental phasing signals are available to improve phases and remove model bias (e.g., MR-SAD).
44. Other validation tools like molprobity (http://molprobity.manchester.ac.uk) and PROCHECK (https://www.ebi.ac.uk/thornton-srv/software/PROCHECK/) can also be used to check the quality of the final model.
45. The described protocols can also be used for crystal structure determination of sortases by removing transmembrane or flexible regions at the N- and/or C-terminal end identified through bioinformatic analysis. Here, obtaining a structure solution by MR is straightforward since sortases share a common catalytic domain with a conserved fold.

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Chapter 15

Tracking Cell Wall-Anchored Proteins in Gram-Positive Bacteria

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Abstract

Cell wall-anchored surface proteins are integral components of Gram-positive bacterial cell envelope and vital for bacterial survival in different environmental niches. To fulfill their functions, surface protein precursors translocate from cytoplasm to bacterial cell surface in three sequential steps: secretion across the cytoplasmic membrane, covalently anchoring to the cell wall precursor lipid II by sortase A, and incorporation of the lipid II-linked precursors into mature cell wall peptidoglycan. Here, we describe a series of immunofluorescence microscopy methods to track the subcellular localization of cell wall-anchored proteins along the sorting pathway. While the protocols are tailored to *Staphylococcus aureus*, they can be readily adapted to localize cell wall-anchored proteins as well as membrane proteins in other Gram-positive bacteria.

Key words Immunofluorescence microscopy, Cell wall-anchored proteins, *Staphylococcus aureus*, Protein A (SpA), Surface display, Cross-wall, Septum

1 Introduction

Cell wall-anchored proteins are key components of the Gram-positive bacterial cell envelope. These proteins are covalently attached to cell wall peptidoglycan, displayed on bacterial cell surface, and are vital for bacterial interaction with host and survival in different environmental niches [1, 2]. To be displayed on the cell envelope, surface protein precursors undergo three steps: secretion, anchoring, and incorporation into the cell wall. Surface protein precursors are produced in the cytoplasm with an N-terminal signal peptide and a C-terminal cell wall sorting signal [3]. The N-terminal signal peptide directs the precursors to be secreted across the membrane. Following membrane translocation, the signal peptide is cleaved off by the signal peptidase, and the C-terminal cell wall sorting signal retains the precursor to the membrane [4]. The membrane-bound sortase A (SrtA) recognizes the LPXTG

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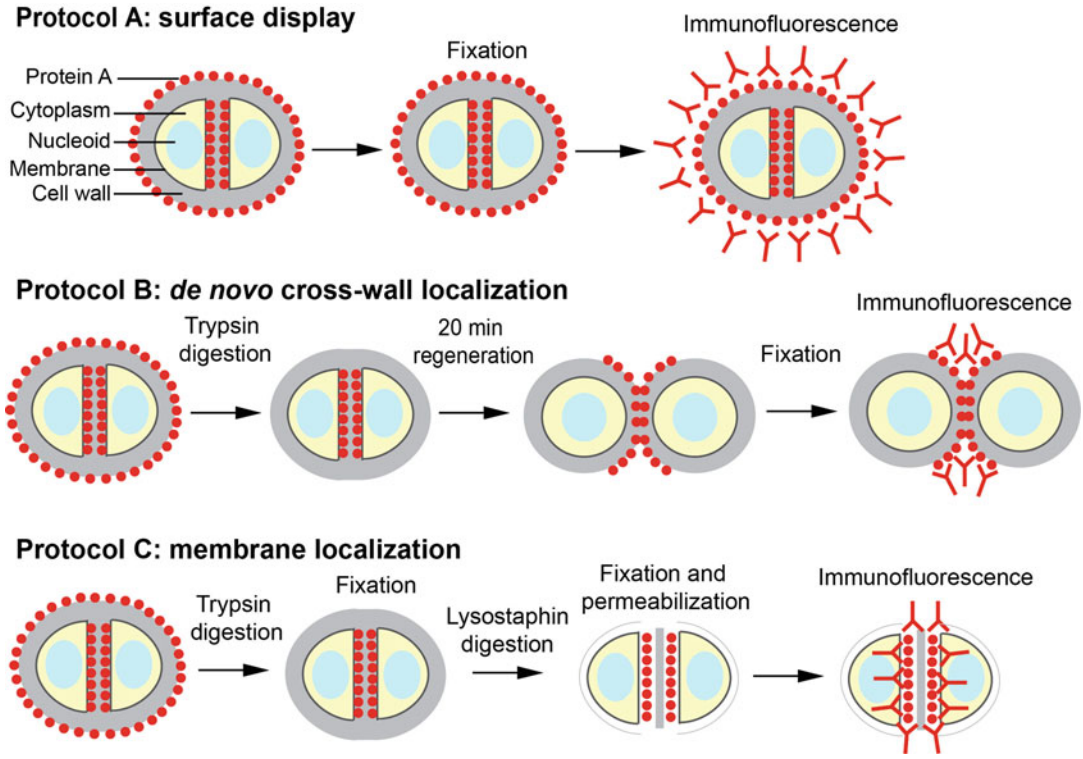

Fig. 1 Schematic diagram of the protocols. Note that while the newly anchored SpA localizes at the cross-wall, other surface proteins may have different localization patterns. (Reproduced from Ref. [20])

motif in the cell wall sorting signal, cleaves the amide bond between threonine (T) and glycine (G), and covalently links the surface protein precursor to the pentaglycine cross-bridge of peptidoglycan precursor lipid II [5]. In the final step, the lipid II-linked surface protein precursor is incorporated into the mature cell wall peptidoglycan via transglycosylation and transpeptidation during cell wall synthesis [5, 6].

Historically, the sorting pathway has been well studied by biochemical methods [5–13]. Recent cell biological studies show that surface proteins have specific subcellular localizations that are associated with their biological functions [14–18]. Here, we describe a series of immunofluorescence microscopy methods (protocol A, B, C) to reveal the subcellular localizations of surface protein in *Staphylococcus aureus* (Fig. 1). The model protein we use is protein A (SpA), an abundant cell wall-anchored protein in *S. aureus* that binds to host immunoglobulin and triggers immune evasion [19]. Protocol A is termed "surface display", in which bacterial cells are harvested and fixed at the desired growth phase, stained with primary antibodies against a specific surface protein antigen (e.g., SpA in our experiment), and subsequently stained with fluorescent-labeled secondary antibodies. "Surface display"

detects specific proteins on the bacterial cell surface. Protocol B, termed "de novo cross-wall localization", utilizes a "pulse-chase" labeling method: bacterial cells are first treated with trypsin to remove all surface-exposed proteins (trypsinization); trypsin-treated cells are washed and re-grown in fresh media to allow the deposition of newly synthesized surface proteins to the bacterial cell surface (regeneration); the cells are then fixed and stained with primary and fluorescent secondary antibodies. This method detects the localization of newly anchored surface proteins. Protocol C is termed "membrane localization". In this protocol, staphylococcal cells are fixed and treated with the cell wall hydrolase lysostaphin, to generate protoplasts; the protoplasts are fixed for immunofluorescence labeling to detect proteins at the membrane compartment; when needed, the cytoplasmic membrane can be permeabilized with Triton X-100 to allow the detection of cytoplasmic proteins. While these protocols are specifically tailored to *S. aureus*, the methods can be modified to track surface proteins in other Gram-positive bacteria. Furthermore, protocol C is not restricted to surface proteins; it can also be used to localize cytoplasmic or membrane proteins. The protocols are adapted from Ref. [20] with modification.

2 Materials

2.1 Bacterial Sample Preparation

Prepare all solutions with ultrapure water and filter-sterilize. Stock solutions are stored at −20 °C and working solutions at 4 °C, unless otherwise indicated.

1. Tryptic Soy Broth (TSB): Dissolve 30 g of TSB powder in 1 L ddH_2O, autoclave at 121 °C for 45 min.
2. Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM K_2HPO_4, pH 7.4. Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g K_2HPO_4 in 1 L ddH_2O. Adjust pH to 7.4 with HCl and autoclave at 121 °C for 20 min. Store at room temperature.
3. Fixation solution: 2.4% paraformaldehyde and 0.01% glutaraldehyde in PBS. Prepare stock solutions of glutaraldehyde 50% in H_2O and paraformaldehyde 4% in PBS. Mix 30 mL 4% paraformaldehyde and 10 μL 50% glutaraldehyde, then add PBS to a 50 mL total volume. Store at 4 °C.
4. GTE solution: 50 mM glucose, 20 mM Tris-HCl, 10 mM EDTA, pH 7.5. Make stock solutions of 0.5 M EDTA (pH 8) and 1 M Tris-HCl (pH 7.5). Add 0.9 g of D-glucose, 2 mL of 0.5 M EDTA, and 2 mL of 1 M Tris-HCl to a final volume of 80 mL of ddH_2O. Adjust pH to 7.5 with HCl, add ddH_2O to 100 mL, filter-sterilize and store at 4 °C.

5. 5 mg/mL trypsin stock in PBS, filter-sterilized and stored at −20 °C.
6. 10 mg/mL trypsin inhibitor in ddH_2O, filter-sterilized and stored at −20 °C.
7. 10 mg/mL Lysostaphin stock in 20 mM sodium acetate, pH 4.5 stored at −20 °C; for a 2 mg/mL working solution, dilute with 200 mM Tris-HCl, pH 8 and store at 4 °C.
8. 0.2% TritonX-100 in PBS.

2.2 Immunofluorescence Staining

1. 8-well glass slides and rectangular cover glasses No.1 (22 × 50 mm).
2. 0.1% poly-L-lysine solution.
3. Primary antibody: SpA_{KKAA} antiserum [21], store at 4 °C.
4. Secondary antibodies: Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 or Alexa Fluor 488 labeled; store at 4 °C.
5. Molecular Probes™ SlowFade™ Diamond Antifade Mountant.
6. Bovine Serum Albumin (BSA) blocking solution: 3% BSA in PBS. Dissolve 0.3 g of BSA in 10 mL PBS, make it freshly and filter-sterilize before use, store at 4 °C for use within a week.
7. 10 mg/mL Hoechst 33342 DNA dye, store at 4 °C.
8. Nile Red: make a stock solution of 0.5 mg/mL in 100% ethanol, store at −20 °C. Add 5 μL Nile Red stock solution to 1 mL PBS (1:200 dilution) to stain the samples.
9. BODIPY™ Vancomycin-FL (Van-FL): Dissolve 100 μg in 100 μL DMSO to make a 1 μg/μL stock solution, store at −20 °C. Add 2 μL of Van-FL stock solution to 1 mL of PBS (1:500 dilution) to stain the samples.

3 Methods

3.1 Bacterial Cultures

1. Prepare an overnight culture by inoculating one single colony to 3 mL TSB in a test tube; add appropriate antibiotics when needed.
2. Grow the overnight culture at 37 °C with rigorous shaking.
3. The next morning, dilute the overnight culture to fresh TSB to start the main culture (inoculate 30 μL overnight culture to 3 mL fresh TSB, 1:100 dilution).
4. Grow the main culture at 37 °C with rigorous shaking until OD_{600} reaches 0.8–1.0 (*see* **Note 1**).
5. Continue with sample preparation in Subheading 3.2.

3.2 Sample Preparation

3.2.1 Slide Preparation

1. Add 50 µL of 0.1% poly-L-lysine to each well of an 8-well glass slide and incubate for 5 min at room temperature (*see* **Note 2**).
2. Briefly rinse the wells with ddH_2O, remove the excess liquid using a vacuum, and allow the slide to dry completely (*see* **Note 3**).

3.2.2 Surface Display

1. Transfer 2 mL bacterial culture into a 2 mL microcentrifuge tube.
2. Spin at 18,000 × *g* for 3 min in a tabletop centrifuge to pellet the bacterial cells.
3. Remove the supernatant without disturbing the pellet.
4. Resuspend the pellet in 1 mL of PBS and vortex well.
5. Spin at 18,000 × *g* for 3 min and remove the supernatant (**steps 4** and **5** are "wash with PBS" steps).
6. Resuspend the pellet in 1 mL of PBS, vortex thoroughly; mix 250 µL of bacterial suspension with 250 µL of fixation solution in a clean 1.5 mL microcentrifuge tube, briefly vortex to mix, and incubate for 20 min at room temperature (fixation step).
7. Wash twice with PBS, as described in **steps 4** and **5** (*see* **Note 4**).
8. Resuspend the pellet in 150 µL of PBS, vortex thoroughly (*see* **Note 5**).
9. Add 50 µL of bacterial suspension to poly-L-lysine-coated glass slides and allow sitting for 5 min.
10. Remove the excess liquid (non-adherent cells) using a vacuum.
11. Add one drop of PBS (about 25 µL) to each well using a plastic disposable transfer pipette and remove using a vacuum (this is the "drop and remove on-slide wash" step).
12. Repeat the drop and remove on-slide wash step.
13. Continue with immunofluorescence staining in Subheading 3.3.

3.2.3 De Novo Cross-wall Localization

1. Transfer 2 mL of bacterial culture into a 2 mL microcentrifuge tube.
2. Spin at 18,000 × *g* for 3 min in a tabletop centrifuge to pellet the bacterial cells.
3. Remove the supernatant without disturbing the pellet.
4. Wash once with PBS.
5. Resuspend the pellet in 900 µL of PBS, vortex thoroughly; add 100 µL of 5 mg/mL trypsin stock solution and briefly vortex (trypsin final concentration of 0.5 mg/mL).

6. Incubate the tubes in a mini-tube rotator at 37 °C for 1 h at a gentle rotation speed of 16.
7. Wash twice with PBS.
8. Resuspend the pellet in 900 μL of fresh TSB, vortex thoroughly; add 100 μL of 10 mg/mL soybean trypsin inhibitor stock solution and briefly vortex to mix (final concentration of soybean trypsin inhibitor of 1 mg/mL).
9. Incubate the tubes in a mini-tube rotator at 37 °C for exactly 20 min at a rotation speed of 16 (*see* **Note 6**).
10. Add 250 μL of fixation solution to a clean 1.5 mL microcentrifuge tube during the 20 min incubation.
11. At the 20 min time point, quickly transfer 250 μL bacterial sample to the microcentrifuge tubes containing fixation solution prepared in the previous step.
12. Vortex to mix and incubate at room temperature for 20 min.
13. Wash twice with PBS.
14. Resuspend the pellet in 150 μL of PBS and vortex thoroughly (adjust the volume depending on the pellet size).
15. Add 50 of μL of bacterial suspension to a glass slide coated with poly-L-lysine and allow sitting for 5 min.
16. Remove the liquid (non-adherent cells) using a vacuum.
17. Perform the drop and remove on-slide wash twice for each well as described above.
18. Continue with immunofluorescence staining in Subheading 3.3.

3.2.4 Membrane Localization

1. Place two 50 mL tubes containing approximately 25 mL methanol and 25 mL acetone, respectively, into a −20 °C freezer for at least an hour.
2. Normalize all the bacterial cultures to $OD_{600} = 1$.
3. Transfer 2 mL of normalized bacterial culture to a 2 mL microcentrifuge tube (*see* **Note 7**).
4. Spin at 18,000 × *g* for 3 min in a tabletop centrifuge to pellet the bacterial cells.
5. Remove the supernatant without disturbing the pellet.
6. Wash once with PBS.
7. Resuspend the pellet in 900 μL of PBS and vortex thoroughly; add 100 μL of 5 mg/mL trypsin stock solution and briefly vortex (*see* **Note 8**).
8. Incubate in a mini-tube rotator at 37 °C for 1 h at a rotation speed of 16.
9. Wash twice with PBS.

10. Resuspend the pellet in 500 μL of PBS, vortex thoroughly; add 500 μL of fixation solution and vortex to mix.
11. Incubate the sample for 15 min at room temperature and then on ice for 15 min to fix.
12. Wash three times with PBS.
13. Resuspend the pellet in 1 mL of freshly made GTE buffer and vortex thoroughly (*see* **Note 9**).
14. Add 50 μL of cell suspension to poly-L-lysine-coated glass slides (this is the control without lysostaphin digestion) (*see* **Note 10**).
15. Prepare a timer, add 10 μL of lysostaphin working solution to the rest of the cell suspension; quickly vortex and immediately add 50 μL to the glass slides (*see* **Note 11**).
16. Incubate for 2 min on the slide (*see* **Note 12**).
17. Remove the liquid using a vacuum until completely dry.
18. Immediately place the slide into prechilled methanol at −20 °C for 5 min.
19. Take out the slide using forceps and place into prechilled acetone at −20 °C for 30 s (*see* **Note 13**).
20. Take out the slide using forceps and allow to air-dry completely (*see* **Note 14**).
21. Once the slide is dry, apply 50 μL PBS to the sample well to rehydrate.
22. Perform the drop and remove on-slide wash twice for each well.
23. Continue with immunofluorescence staining in Subheading 3.3.

3.3 Immunofluorescence Staining

1. Remove PBS, add BSA blocking solution, and incubate for 30 min at room temperature (or overnight at −4 °C).
2. Remove the blocking solution, add 50 μL primary antibody solution (rabbit serum SpA_{KKAA} 1:4000 dilution in BSA blocking solution), and incubate overnight at 4 °C or at room temperature for 1 h (*see* **Note 15**).
3. Remove the unbound primary antibody solution and wash 8 times with PBS using a vacuum with the last wash step for 5 min.
4. Remove the washing solution, add 50 μL secondary antibody diluted in BSA blocking solution (e.g., Alexa Fluor 647-IgG or Alexa Fluor 488-IgG, 1:500 dilution), and incubate in the dark for 1 h at room temperature (*see* **Note 16**).
5. Perform the on-slide wash 10 times with PBS.

6. Take a clean 1.5 mL microcentrifuge tube, add 1 mL of PBS, 5 μL of Hoechst stock solution (1:200 dilution), 2 μL of Van-FL stock solution (1:500 dilution), or 5 μL of Nile Red stock solution (1:200 dilution) and mix well; add 50 μL of staining solution to each well.
7. Incubate in the dark for 10 min at room temperature.
8. Perform the on-slide wash three times with PBS.
9. After the last wash, remove all the excess liquid from the well.
10. Add a 5 μL drop of SlowFade™ Diamond Antifade Mountant at 3 different places between the sample wells (*see* **Note 17**).
11. Brush a thin layer of nail polish around the edges of the slide and seal with a cover slip; gently press the cover slip and use a Kimwipes to remove the excess antifade solution around the edges (*see* **Note 18**).
12. The prepared slides can be stored at 4 °C for a few days and at −20 °C for a longer period; however, immediate imaging is recommended.

3.4 Imaging and Data Analysis

1. The samples prepared above are suitable to be imaged by different imaging systems, including epi-fluorescence microscopy, confocal microscopy, or deconvolution microscopy. To reveal bacterial cellular features and define protein localization in tiny bacterial cells, a microscope with high resolution is recommended. A 60× or 100× objective lens with a higher numerical aperture is needed. We used a Leica DM 2000 epi-fluorescence microscopy coupled with a sensitive CCD camera, a Leica SP5 2-photon Laser Scanning Confocal microscope, a Leica SP8 3× STED Laser Confocal Microscope, and a GE Applied Precision DeltaVision Elite deconvolution fluorescence microscope equipped with a Photometrics CoolSnap HQ2 camera All showed good imaging results [22, 23].
2. For imaging, adjust microscope settings based on positive and negative controls. Once the parameters are set, use the same settings to capture images of all samples. Take at least three images with random views for each sample in each experiment. Perform at least three times of independent experiments.
3. To quantify the frequency of SpA cross-wall localization, count the number of cells with cross-wall SpA localization and the total number of cells from the same image using "cell counter" tool in Image J software [24] (Rasband W.S./U. S. NIH, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/). To quantify the intensity of SpA signals at the cross-wall or septum, draw a line at the middle of the cell and perpendicular to cross-wall or septum in Image J, which generates a histogram of fluorescence intensity along the line. The relative cross-wall

signal intensity is the ratio of cross-wall signals versus the peripheral signals of the same cell. Minimum 50 cells per sample per experiment were taken for quantification. Three independent experiments were performed to analyze statistical difference.

4. Use GraphPad Prism Software for statistical analysis (https://www.graphpad.com/scientific-software/prism/). In GraphPad Prism, use a t-test to statistically analyze significant differences between two groups; use one-way ANOVA for multiple group comparisons; and use Tukey's multiple comparison test to analyze differences among multiple groups.

4 Notes

1. The growth conditions and the time to harvest bacteria should be modified according to the different proteins and strains. For example, the expression pattern of surface proteins can be different: some are more abundantly expressed in log phase whereas others are expressed in stationary phase. SpA is known to be highly expressed in log phase, thereby we harvest the bacteria in log phase.
2. This step is to coat the slides with poly-L-lysine to mobilize the bacterial cells. A 1:10 dilution of poly-L-lysine to 0.01% also works. We use multi-well slides to process multiple samples on one slide, but one can use different types of glass slides and cover slips. The thickness of the cover slip should fit the imaging system.
3. A bench vacuum system is needed to efficiently wash the samples. In our lab, we assemble a simple bench vacuum system as follows: connect an in-house vacuum to a tube, cut the extremity off a 200 μL pipette tip, and insert the tip into the tube. It is important not to touch the samples on the glass slides during drying.
4. Cells tend to clump after fixation; a longer vortex may be needed.
5. It happens often to first-time user of this protocol that bacterial pellet disappear after washing. There is no need to panic if this happens, as there are probably sufficient bacteria cells for imaging even if no pellet can be seen. The volume of PBS added can be adjusted according to the pellet size; even if no pellet can be seen, one can add a very small volume of PBS (e.g., 20 μL). If the pellet is big, one should use more PBS. The key point here is to have a proper number of cells on the slide, so that most of the cells are well separated. Too many cells will lead to bacterial

clumping and cause artifacts in immunostaining; too few cells will not provide reliable results.

6. The time of re-generation was determined experimentally in our protocol. It should be tested and optimized depending on different growth conditions and antigens.
7. This step is to have the same cell numbers for the following enzymatic digestion step.
8. This step is not critical for protocol C, as lysostaphin digestion will remove most of the cell wall as well as the existing SpA. We include this step in our protocol to minimize any potential background caused by existing SpA.
9. GTE buffer is an osmotic stabilizing buffer. Lysostaphin is a zinc-dependent endopeptidase [25]. Although EDTA in the GTE buffer can chelate zinc, it does not have any obvious negative effects in our experiments. We have tried other osmotic stabilizing buffers without EDTA, such as TSM [50 mM Tris-HCl (pH 7.5), 0.5 M sucrose, 10 mM $MgCl_2$], which also works.
10. It is important to have this control. Staphylococcal cells after lysostaphin digestion will become more translucent in bright-field images, whereas undigested cells have a dark cell contour.
11. Other cell wall hydrolases can substitute lysostaphin if this protocol is to be adapted for another bacterium. Lysozyme, for example, has been used in *Bacillus subtilis* [26, 27]. Most *S. aureus* strains are lysozyme-resistant, which limits its use in *S. aureus*. The digestion time and buffer will have to be adjusted experimentally for a different enzyme or bacterium.
12. It is critical to perform on-slide digestion to stabilize the protoplasts.
13. This step is critical; it stabilizes the protoplast after lysostaphin digestion and permeabilizes the cytoplasmic membrane.
14. To detect cytoplasmic proteins, Triton X-100 can be added here to further permeabilize the cytoplasmic membrane. We used 0.2% TritonX-100 and incubated for 5 min. However, the concentration and incubation time should be determined experimentally depending on the bacterial strain and abundance of antigens.
15. For any new antigen, serial dilution of primary antibody is necessary to determine the optimal concentration. A negative control that does not express the antigen is an essential control. If there is no mutant available, one should include at least a control without primary antibody. Minimal background signals should be seen in the negative control.

16. Depending on the microscope system, one can choose different fluorescent-labeled secondary antibodies. We consistently use the secondary antibody at a 1:500 dilution.
17. Different kinds of antifade solution are commercially available. One should choose the antifade solution compatible with the imaging system.
18. Make sure that the antifade solution covers every well as a very thin layer without bubbles, and that the cover slip is leveled on the slide.

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Chapter 16

Probing Bacterial Cell Division and Cell Envelope Biogenesis with Live-Cell Fluorescence Microscopy

Lauren R. Hammond, Maria L. White, and Prahathees J. Eswara

Abstract

The development of advanced microscopy techniques has ushered in a new era of research as it helps understand biological processes on a deeper, mechanistic, and molecular level like never before. Live-cell fluorescence microscopy has importantly allowed us to visualize subcellular protein localization and incorporation of various fluorophores compatible with living cells in real time. As such, this technique offers valuable insights at the single-cell level and enables us to monitor phenotypic differences that were easily overlooked at a population level. One area of research that has benefited greatly from these advances is the study of the bacterial cell envelope biogenesis and cell division process. In this report, we provide detailed protocols, optimized in our lab, for imaging these processes in the Gram-positive organisms *Bacillus subtilis* and *Staphylococcus aureus*.

Key words FtsZ, Wall teichoic acids, Concanavalin A, Cell wall, Peptidoglycan, Vancomycin

1 Introduction

The emergence of high-resolution fluorescence microscopy has allowed for rapid developments in the field of bacterial cell biology. The advent of this technique has allowed visualization of fundamental cellular processes in bacteria such as DNA replication and cell division in live cells. Since it was first developed, many new fluorophores have been generated further increasing the possibilities of this technique. Herein, we provide a detailed protocol for timelapse microscopy to track fluorescently tagged proteins and two different staining techniques to visualize the bacterial cell envelope. In our first example we show how we have used timelapse microscopy to track the localization of the central divisome component FtsZ, translationally fused to the green fluorescent protein (GFP), over time when the divisome is perturbed by overproduction of a cell division regulator MraZ in *Bacillus subtilis* [1]. Second, we provide a detailed protocol for using Concanavalin A (ConA)

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conjugated to FITC to stain wall-teichoic acids (WTA), which are anionic glycopolymers that make up to 60% of the Gram-positive cell wall [2]. ConA is a lectin that has been shown to specifically recognize and bind to the carbohydrate moiety of WTA [3]. This protocol has been adapted from previous studies and optimized for staining WTAs in the Gram-positive organisms *B. subtilis* and *Staphylococcus aureus* [4–8]. Finally, we show how to use fluorescently tagged vancomycin (FL-Vanc) for labeling the peptidoglycan cell wall in *S. aureus*. Vancomycin is a type of glycopeptide antibiotic that tightly binds to the terminal D-Ala-D-Ala of the uncrosslinked pentapeptide of the peptidoglycan precursor [9, 10]. Previous work successfully demonstrated the utility of this technique in *B. subtilis* and *S. aureus* [11–14], here we provide a detailed protocol optimized for deconvolution microscopy. Video demonstration of several key imaging steps was published previously [15].

2 Materials

1. Lysogeny Broth (LB) (for *Bacillus subtilis*): suspend 10 g tryptone, 10 g yeast extract, and 10 g NaCl to 1 L of H_2O and autoclave for sterilization.
2. Tryptic Soy Broth (TSB) (for *Staphylococcus aureus* use): suspend 17 g tryptone, 3 g soytone, 2.5 g dextrose, 5 g NaCl, and 3.28 g $K_2HPO_4.3H_2O$ to 1 L of H_2O and autoclave for sterilization.
3. *Bacillus* strains PY79 (wildtype) and MW205 ($P_{hyperspank}$-*mraZ spc*, *ftsAZ::ftsAZ-gfpΩerm*; [1]).
4. *S. aureus* SH1000 strain.
4. Baffled 125 mL flask and shaking incubator.
5. Spectrophotometer.
6. 1.5 mL microcentrifuge tube – black and clear.
7. Shaking heat block.
8. Microcentrifuge.
9. Glass bottom microscope dish.
10. Agarose for making agarose pads will be dissolved in either LB or sterile H_2O to a concentration of 1%.
11. Petri dish.
12. Concanavalin A-FITC; final working concentration is 200 μg/mL. Lyophilized powder dissolved in 0.9% NaCl.
13. Boron-dipyrromethene (BODIPY; 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) fluorescent dye.
14. Vancomycin.

15. SynaptoRed Membrane Dye; final working concentration is 1 μg/mL, dissolved in H_2O.
16. Isopropyl β-D-1-thiogalactopyranoside (IPTG): 10 mM working concentration dissolved in H_2O.
17. Fluorescence microscope equipped with necessary filters (FITC, TRITC, and DIC) and deconvolution software.

3 Methods

3.1 Timelapse Microscopy

See Fig. 1a for Timelapse microscopy workflow.

3.1.1 Growth Conditions

1. Grow a 2 mL overnight culture of *B. subtilis* in LB.
2. The following day, dilute the overnight culture into a baffled flask with 10 mL of LB to an OD_{600} of 0.1.
3. Grow to exponential phase (OD_{600} of 0.4–0.6).
4. Dilute a second time into 10 mL of LB to an OD_{600} of 0.1.
5. Grow to desired growth phase (*see* **Note 1**).

3.1.2 Preparation of Samples for Timelapse Microscopy

1. Make 1% agarose in lysogeny broth (LB) by dissolving 0.2 g of agarose in 20 mL of LB. Pour into a Petri dish to solidify.
2. Cut an agarose pad of approximately 11 mm in diameter (*see* **Note 2**).
3. Place 5 μL of culture on the center of a MatTek glass bottom dish and cover with the LB agarose pad.
4. Gently press down the agarose pad (*see* **Note 3**).
5. Add a few drops of water around the sample to maintain humidity in the microscope dish (*see* **Note 4**).
6. Allow sample to sit in the microscope chamber for 30 min prior to imaging.
7. Add 10 μL of 10 mM IPTG inducer to the top of the agarose pad and then begin the imaging procedure after 15 min of stabilization.

3.1.3 Imaging Procedure

1. Once the sample is in focus on the microscope use the Point List function to record 3–4 fields of interest for imaging.
2. Select the appropriate channels for imaging (FITC and POL/DIC).
3. Set image acquisition for 5 Z-stacks 200 nm apart.
4. Use the timelapse settings to set the intervals and duration of the imaging, for example, image every 15 min for 2 h (*see* **Notes 5** and **6**).

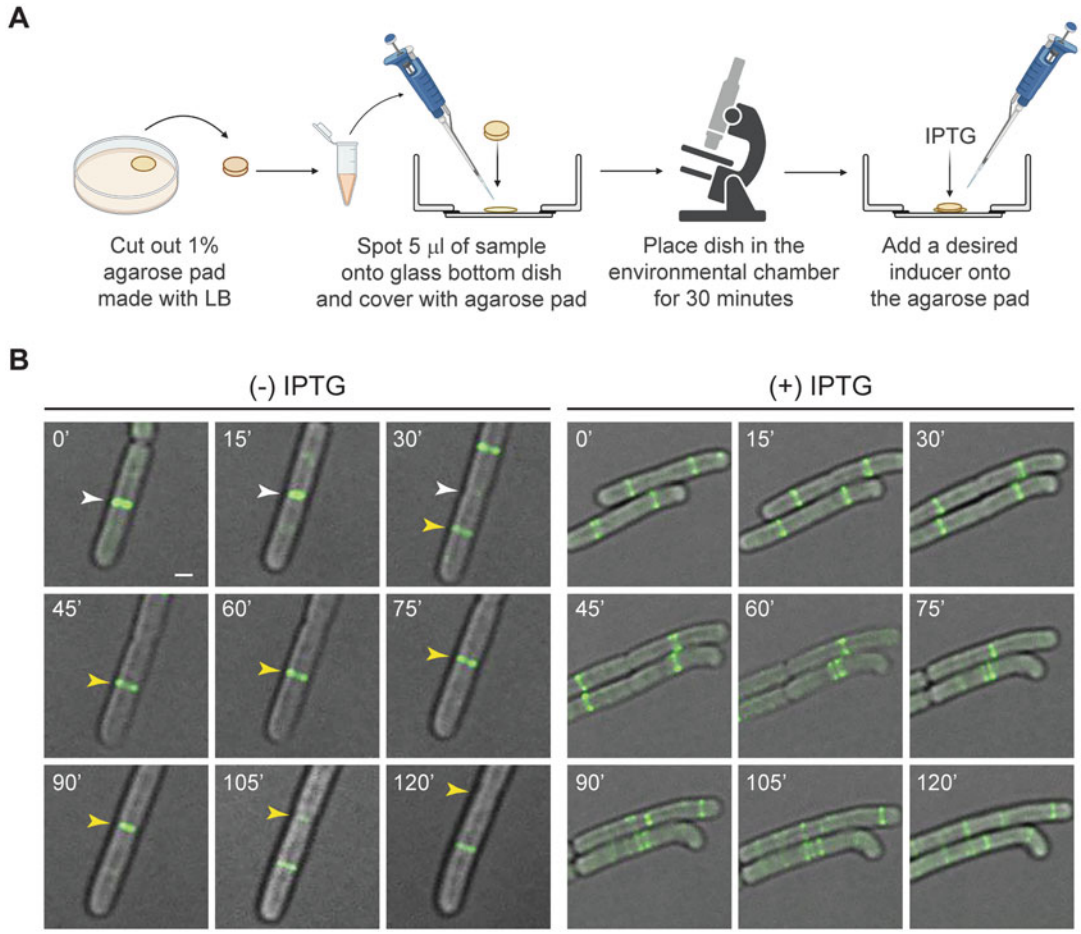


Fig. 1 (**a**) Cut out disk of 1% agarose made with lysogeny broth. Spot 5 μL of culture onto microscope dish and cover with agarose pad. Incubate sample in environmental chamber for at least 30 min. If needed, add IPTG or a desired inducer to the top of the agarose pad. (**b**) Timelapse microscopy of a *B. subtilis* strain harboring *ftsZ-gfp* under the control of its native promoter and an IPTG-inducible copy of *mraZ* (MW205). Images were taken at 15-min interval for 2 h using the FITC (GFP) and DIC channels. Overlay of both channels is shown. The green band indicates the ring-like localization of FtsZ-GFP at sites of cell division. Arrowheads are added to aid in tracking corresponding division sites. Overproduction of MraZ (presence of IPTG) leads to FtsZ ring disassembly [1]. Scale bar is 1 μm. (**a**) was made with the help of Biorender.com

5. Start the timelapse (*see* **Note 7**).
6. Once imaging is complete deconvolve all images using SoftWorx software (*see* Fig. 1b; **Note 8**).

3.2 Fluorescein Labeled Concanavalin A WTA Staining

See Fig. 2a for a diagram of ConA-FITC staining workflow.

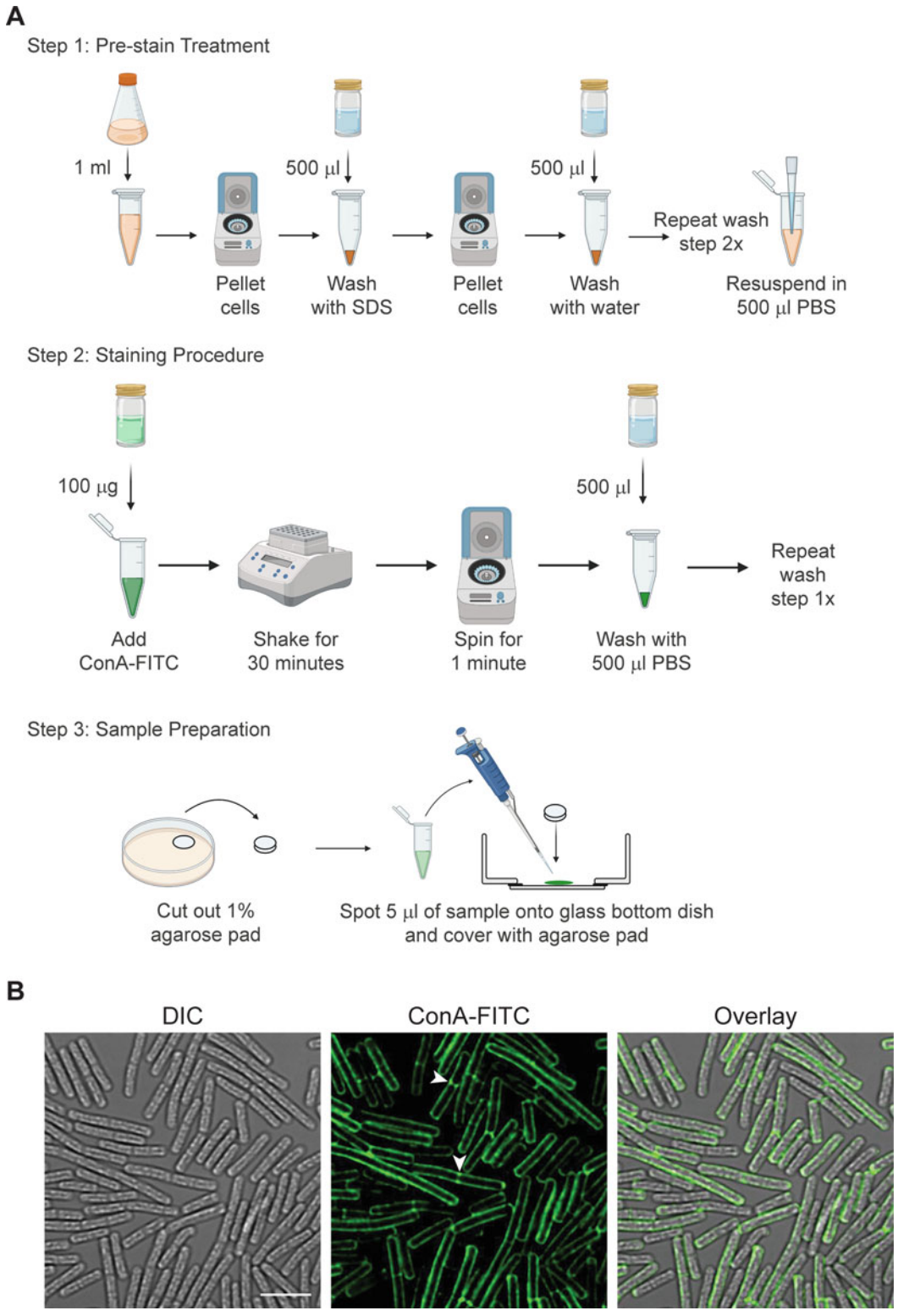


Fig. 2 Workflow for imaging concanavalin A-FITC stained *B. subtilis*. (**a**) Step 1: Wash cells with 10% SDS. Step 2: Stain with ConA-FITC, incubate sample, and wash with PBS. Step 3: Spot sample onto dish for imaging and cover with agarose pad. (**b**) Micrographs showing wildtype *B. subtilis* (PY79) cells stained with ConA-FITC. Arrowheads indicate enrichment of ConA staining at division sites. Scale bar is 15 µm. (**a**) was made using Biorender.com

3.2.1 Pre-Stain SDS Treatment

1. Grow *B. subtilis* as described in Subheading 3.1.1.
2. Take 1 mL of grown culture and transfer to 1.5 mL microcentrifuge tube.
3. Pellet the cells at 5000 RPM (~2000 × g) for 1 min.
4. Resuspend cells in 500 µL of 10% SDS (*see* **Note 9**).
5. Pellet the cells.
6. Wash cells in 500 µL of sterile H_2O or PBS.
7. Repeat wash 2 more times.
8. After the last wash, resuspend pellet in 500 µL PBS.

3.2.2 Concanavalin A-FITC Staining

1. Add 100 µg (200 µg/mL) of ConA-FITC to sample.
2. Incubate with shaking at room temperature for 30 min.
3. Pellet cells and wash with 500 µL PBS twice.
4. Discard supernatant and resuspend cells in residual PBS (*see* **Note 10**).

3.2.3 Sample Imaging

1. Prepare agarose pad as detailed in Subheading 3.1.2.
2. Spot 5 µL of stained sample onto the bottom of a glass bottom dish and cover with the agarose pad.
3. Gently press down the agarose pad.
4. Image using the DIC and FITC channels acquiring 17 Z-stacks 200 nm apart.
5. Deconvolve images with SoftWorx software (*see* Fig. 2b).

3.3 Fluorescent Vancomycin-Based Peptidoglycan Staining

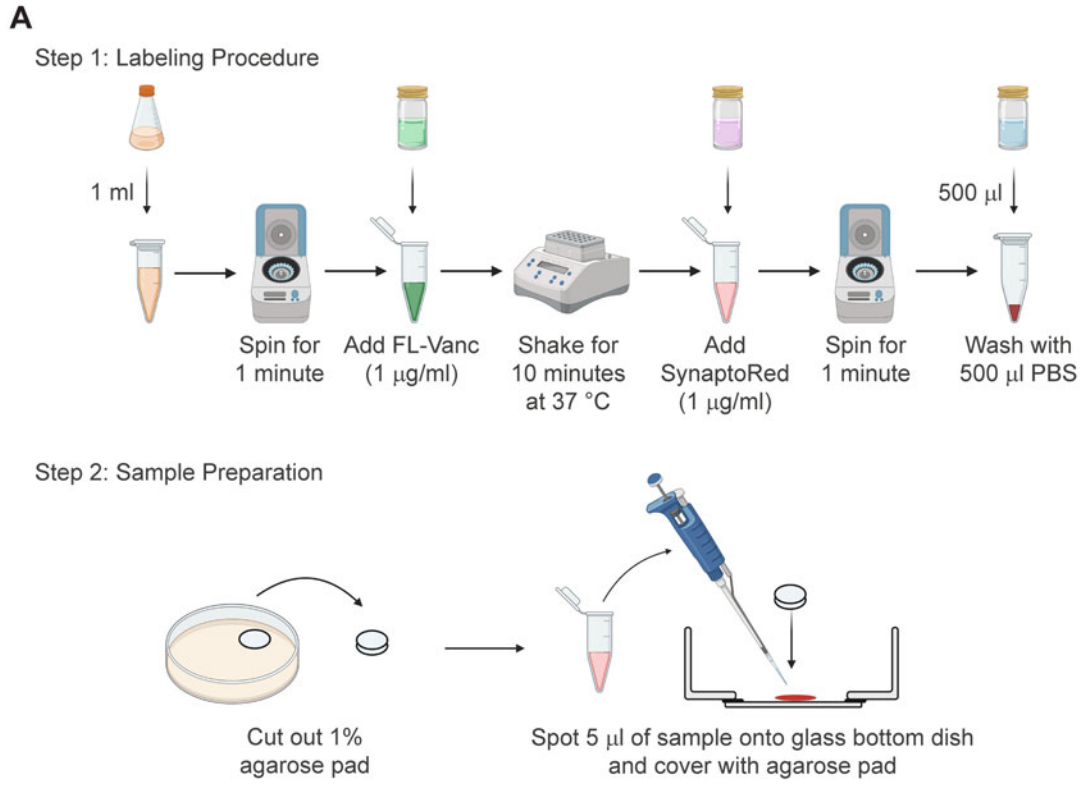
See Fig. 3a for a diagram of BODIPY staining workflow.

3.3.1 Growth Conditions of Staphylococcus Aureus

1. Grow a 2 mL overnight culture of *S. aureus* in TSB.
2. The following day, dilute the overnight culture into a baffled flask with 10 mL of TSB to an OD_{600} of 0.1.
3. Grow to desired growth phase.

3.3.2 BODIPY Staining Procedure

1. Mix BODIPY dye (light sensitive) with vancomycin in a 1:1 ratio (*see* **Note 11**).
2. Pellet the cells at 5000 rpm (~2000 × g) in a microcentrifuge tube for 1 min.
3. Discard supernatant and resuspend pellet in 100 µL of residual media.
4. Add the prepared BODIPY/Vancomycin mixture to the cells to a concentration of 1 µg/mL (*see* **Notes 12** and **13**).
5. Incubate for 10 min.



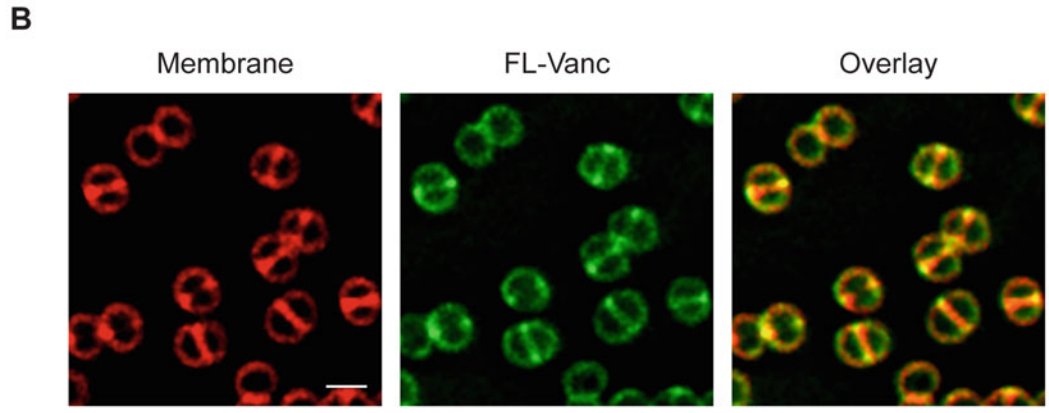


Fig. 3 Workflow for imaging fluorescent BODIPY-conjugated vancomycin (FL-Vanc; *see* **Note 11**) stained *S. aureus* cells. (**a**) Step 1: Pellet 1 ml of culture, label with FL-Vanc (1 µg/mL), incubate, add SynaptoRed membrane dye (1 µg/mL), pellet and wash cells with PBS. Step 2: Spot sample onto dish for imaging and cover with agarose pad. (**b**) Micrographs of *S. aureus* SH1000 cells labeled with FL-Vanc. Scale bar is 1 µm. Peptidoglycan is labeled by FL-Vanc (green) and the membrane is stained with SynaptoRed (red). (**a**) was made with the help of Biorender.com

6. Add SynaptoRed Membrane Dye to a concentration of 1 μg/mL.
7. Pellet cells and wash with 500 μL PBS.
8. Pour off supernatant and resuspend in residual PBS (*see* **Note 10**).

3.3.3 Sample Imaging *See* Subheading 3.2, **step 3** (*see* Fig. 3b).

4 Notes

1. Desired growth phase for timelapse microscopy is typically mid-exponential phase to ensure cells are actively growing and dividing.
2. To create the circular agarose pad that is around 11 mm, we use the bottom of a sterile 2.0 mL cryogenic vial (Corning).
3. Applying slight pressure to the agarose pad is needed to disperse the bacteria, create a monolayer, and prevent movement from motile bacteria (*B. subtilis*).
4. Turning on the microscope incubator the night prior and keeping a small beaker of water inside the chamber helps maintain the humidity within the chamber and keep the cells from drifting.
5. The number of Z-stacks and frequent imaging contribute to phototoxicity.
6. Many of the staining procedures used for fluorescence microscopy can be phototoxic to the sample. Therefore, during timelapse microscopy careful consideration on whether to stain the sample is important.
7. To ensure that the cells stay in focus throughout the timelapse imaging, check each point is in focus prior to the imaging interval and re-save the point.
8. All images were taken on a DeltaVision Elite microscope system (Leica Microsystems) equipped with a Photometrics CoolSnap HQ2 camera and an environmental chamber. For timelapse microscopy (*see* Fig. 1), to limit potential phototoxicity, five planes were acquired every 200 nm. For staining experiments (*see* Figs. 2 and 3) seventeen planes were acquired. Data was deconvolved using SoftWorx software.
9. Pre-stain SDS treatment for *B. subtilis* is optional (*see* Subheading 3.2.2).
10. Prior to imaging samples there is a wash (PBS) step. The amount of liquid needed to resuspend the pellet will need to be adjusted for the density of the starting culture.

For mid-log cultures (OD_{600} of 0.4–0.6) resuspending in the residual supernatant (~100 μL) works well for both *B. subtilis* and *S. aureus*.

11. BODIPY is light sensitive, we use a black 1.5 mL tube to limit light exposure.
12. To generate FL-Vancomycin, mix BODIPY in a 1:1 ratio with vancomycin and then use at a final concentration of 1 μg/mL. For example, 100 μg of lyophilized BODIPY is resuspended in 1 mL sterile deionized double-distilled H_2O. In a black 1.5 mL centrifuge tube, 50 μL of the BODIPY is mixed with 50 μL of vancomycin (100 μg/mL) to create fluorescently labeled vancomycin (FL-Vancomycin). FL-Vancomycin (1 μL) is then added to a 100 μL sample.
13. Fluorescent D-amino acid labeling could also be used to monitor active incorporation of peptidoglycan precursors [16–18].

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Chapter 17

Assembling the *Bacillus subtilis* Spore Coat Basement Layer on Spherical Supported Lipid Bilayers

Taylor B. Updegrove, Domenico D'Atri, and Kumaran S. Ramamurthi

Abstract

Micro- and nanoparticles are often designed by mimicking naturally occurring structures. Bacterial spores are dormant cells elaborated by some Gram-positive bacteria during poor growth conditions to protect their genetic material from harsh environmental stresses. In *Bacillus subtilis*, this protection is, in part, conferred by a proteinaceous shell, the "coat", which is composed of ~80 different proteins. The basement layer of the coat contains two unusual proteins, which we have recently reconstituted around silica beads to generate synthetic spore-like particles termed "SSHELs". Here, we describe the protocol for generating SSHEL particles, and describe the procedure to covalently link molecules of interest (in this case an anti-HER2 affibody) to SSHEL surfaces. SSHELs therefore represent a versatile platform for the display of ligands or antigens for the site-specific delivery of cargo or vaccines.

Key words SpoVM, SpoIVA, Synthetic biology, Nanoparticle, Drug delivery, Sporulation, *C. difficile*, Cancer

1 Introduction

Certain bacteria initiate a stress response to starvation conditions by metamorphosing into a dormant cell, called an "endospore" (or, simply, a "spore") [1]. Spores are incredibly resistant to harsh environmental conditions [2], but nonetheless resume normal growth when nutrients are again available [3]. This developmental program is called "sporulation" and is limited to a small group of Gram-positive bacteria in the phylum Firmicutes [4]. *Bacillus subtilis* is a genetically tractable, non-pathogenic bacterium that has been a leading model organism to study sporulation, a process that is also conserved in human pathogens such as *Clostridium difficile* and *Bacillus anthracis* [5, 6].

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A hallmark of sporulation is the elaboration of an internal spherical daughter cell, the "forespore", by the rod-shaped bacterium, which contains a copy of the bacterium's chromosome. The forespore then gradually shuts down metabolically as it matures into a spore, whereupon it is released into the environment by the programmed lysis of the outer "mother cell". Part of the maturation of the forespore is the deposition of ~80 different mother cell-produced proteins on the forespore surface that forms a stable shell surrounding the mature spore called the "coat" [7]. The first coat protein to localize to the forespore surface is SpoVM, a 26 amino acid-long amphipathic helical protein that preferentially embeds in positively curved (convex) membranes [8, 9]. SpoVM then recruits the morphogenic protein SpoIVA which uses ATP hydrolysis to drive structural changes that promote the irreversible polymerization of SpoIVA around the forespore [10, 11]. Polymerized SpoIVA provides a sturdy platform for the subsequent deposition of the ~80 proteins that comprise the coat of the mature spore [7].

We previously reconstituted the basement layer of the spore coat using purified SpoVM and SpoIVA that assemble in an ATP-dependent fashion around 1 μm-diameter silica beads coated with a lipid bilayer [12]. We refer to these resulting spore-like particles as synthetic spore husk-encased lipid bilayers, or "SSHELs" (*see* Fig. 1). To modify SSHEL surfaces for use as a delivery platform, we exploited engineered cysteine residues, or primary amine groups, on SpoIVA for chemical modification using "click" chemicals such as *trans*-cyclooctene (TCO) [13]. By utilizing a cognate click chemistry molecule, such as tetrazine, ligated onto various molecules of interest, such as peptides, proteins, or small molecules, the molecule of interest may be covalently displayed on the SSHEL surface via the fast inverse-electron demand Diels-Alder cyclo-addition reaction. SSHELs with ligated molecules can be used for antigen presentation of vaccines or targeted cell therapies against cancer [14, 15]. Here, we detail the protocol for construction of SSHELs with the functional TCO group conjugated to an engineered cysteine residue on the SpoIVA protein that can react to proteins and molecules with the tetrazine functional group. As an example, we present the protocol to display an anti-HER2 affibody (αHER2) on the SSHEL surface.

2 Materials

Prepare all solutions using analytical grade reagents and deionized water that has attained a sensitivity of 18 MΩ-cm at 25 °C. Prepare and store all reagents at room temperature (unless indicated otherwise).

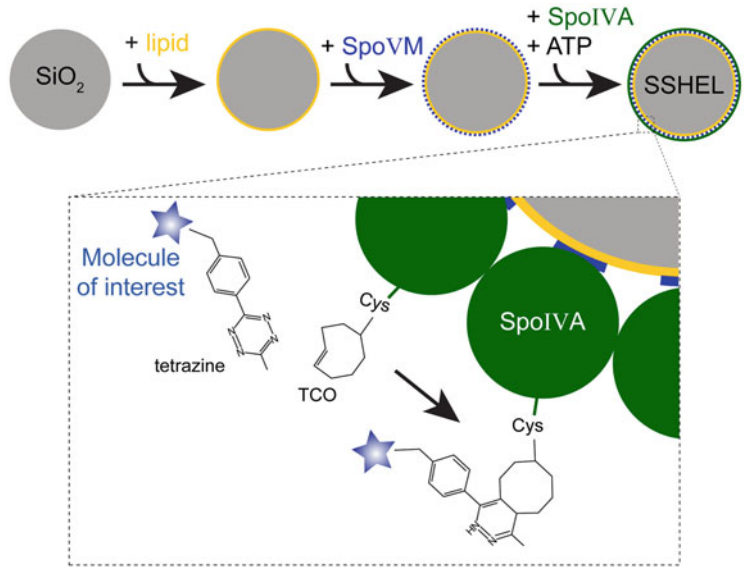

Fig. 1 Schematic of SSHEL assembly. 1 μm diameter silica beads (gray) are covered in a lipid bilayer composed of DOPC (yellow). Next, SpoVM peptide (blue) and SpoIVA (green) are added. In the presence of ATP, SpoIVA polymerizes around the membrane-encased bead, anchored to the bead surface by SpoVM. Inset: SpoIVA can be engineered with a single Cys conjugated with TCO, which can covalently attach to a tetrazine-conjugated molecule of interest (blue star)

2.1 Small Unilamellar Vesicle (SUV) Production

1. 25 mg/mL DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) in chloroform (*see* **Note 1**).
2. Borosilicate glass tubes 16 × 100 mm.
3. Vacuum centrifuge desiccator.
4. Branson Ultrasonic Bath 1800.

2.2 Spherical Supported Lipid Bilayer (SSLB) Production

1. Mesoporous silica beads (1 μm diameter) (Glantreo).

2.3 SpoIVA Purification

1. Luria-Bertani (LB) Broth: 10 g of Yeast Extract, 10 g of NaCl, and 10 g of Peptone from casein.
2. Terrific Broth Modified (Fisher) (*see* **Note 2**).
3. Kanamycin disulfate salt.
4. Isopropyl β-D-1-thiogalactopyranoside (IPTG).
5. Dry incubator shaker.
6. *E. coli* strain BL21 (DE3) Competent Cells.

7. *E. coli* strain BL21 (DE3) containing plasmid pJP120 that expresses His_6-tagged cysteine-less *spoIVA* harboring an engineered N-terminal Cys [12].
8. Lysis buffer: 150 mM NaCl, 50 mM Tris-HCl, pH 7.5.
9. French Pressure Cell Press.
10. Ultracentrifuge and appropriate rotor to accommodate 30 mL sample tubes.
11. Ni^{2+}-NTA Agarose (Qiagen).
12. Wash buffer 1: 20 mM imidazole, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl.
13. Wash buffer 2: 80 mM imidazole, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl.
14. Elution buffer: 250 mM imidazole, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl.
15. PD-10 desalting columns (GE).
16. Fast Protein Liquid Chromatography (FPLC).
17. FPLC MonoQ column.
18. FPLC Buffer A: 50 mM Tris-HCl, pH 7.5.
19. FPLC Buffer B: 50 mM Tris-HCl, pH 7.5, 1 M NaCl.

2.4 SSHELs Preparation

1. SSHEL assembly solution: 50 mM Tris, pH 7.5, 10 μM SpoVM, 4 mM ATP, 10 mM $MgCl_2$, 400 mM NaCl and 1.5 μM SpoIVA-αHER2.
2. Trans-Cyclooctene-PEG3-Maleimide (TCO) (Click Chemistry Tools).
3. Dimethyl Sulfoxide (DMSO).
4. Zeba Spin Desalting Columns, 7 K MWCO, 5 mL (Thermo Scientific).
5. Anti-ErbB2 affibody molecule (Abcam).
6. Methyltetrazine-PEG4-Malemeide (Click Chemistry Tools).
7. Tris-(2-Carboxyethyl)phosphine, Hydrochloride (TCEP).
8. SpoVM peptide (MKFYTIKLPKFLGGIVRAMLGSFRKD) (*see* **Note 3**).
9. Tris Buffer: 50 mM Tris-HCl, pH 7.5, 400 mM NaCl.
10. Phosphate buffered saline (PBS) at pH 7.4.
11. Adenosine 5′-triphosphate disodium (ATP).
12. 1 M $MgCl_2$.
13. Quantum Alexa Fluor 488 MESF kit (Bangs Laboratories).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 SpoIVA Purification

1. Make 4 × 5 mL overnight cultures of *E. coli* BL21 (DE3) pJP120 in LB supplemented with kanamycin (50 μg/mL) for plasmid maintenance and use each 5 mL culture to inoculate 4 × 500 mL of Terrific Broth media supplemented with kanamycin (50 μg/mL).
2. Grow cultures for 2 h in dry shaker set at 37 °C, shaking at 250 rpm.
3. Add IPTG for a final 1 mM concentration and continue growing for 4.5 h.
4. Centrifuge each culture at 4000 × g at 4 °C. Remove and discard supernatant (*see* **Note 4**).
5. Resuspend each of the four pellets on ice with 30 mL ice-cold lysis buffer and disrupt cells by French Press twice using a pressure setting of 1200 psi (*see* **Note 5**).
6. Centrifuge each 30 mL lysate at 30,000 × g for 1 h at 4 °C. Collect supernatant in 50 mL conical tubes and place on ice.
7. During centrifugation, equilibrate 4 mL of Ni^{2+}-NTA agarose in a gravity column with 15 mL ice-cold lysis buffer using gravity flow. Cap the column with resin suspended in ~1 mL lysis buffer and place on ice for future use.
8. Add entire cell supernatant (~120 mL) to Ni^{2+}-NTA agarose column equilibrated with lysis buffer (*see* **Note 6**).
9. Wash column with 50 mL cold wash buffer 1, followed by 4 mL cold wash buffer 2 (*see* **Note 6**).
10. Add 10 mL elution buffer, cap the column, and incubate on ice for 30 min (*see* **Note 7**).
11. While column is incubating, equilibrate 3 × PD-10 desalting columns with ice-cold 15 mL lysis buffer using the manufacturer's instructions.
12. Collect column elution in 3.3 mL aliquots (*see* **Note 6**). Add one 3.3 mL elution to each PD-10 desalting column, previously equilibrated with lysis buffer (**step 11**), and elute with 4 mL cold lysis buffer, collecting 1 mL aliquots in Eppendorf tubes for a total of 12 × 1 mL aliquots (*see* **Note 6**).
13. Using an FPLC, equilibrate the MonoQ column with 10 mL Buffer A (*see* **Note 8**).
14. Apply ~10 mL of desalted Ni^{2+}-NTA agarose-purified SpoIVA to the MonoQ column (*see* **Note 9**) and run the following

stepwise program: first 10 mL of 0% Buffer B; second 10 mL of 15% Buffer B; third 10 mL of 30% buffer B; fourth 10 mL of 40% Buffer B; and fifth 10 mL of 100% buffer B, all at 1 mL/min flow rate with 1 mL fraction collection.

15. Collect fractions 33 through 35, corresponding to the third peak on the A_{280} chromatogram and quantitate molarity using UV-spectrophotometer, measuring A_{280} and using the Beer–Lambert law (SpoIVA MW = 57.71 kDa and $\varepsilon = 44.35$) or Bradford assay using bovine serum albumin (BSA) standards (*see* **Note 6**).
16. Store 100 μL aliquots at −80 °C for future use (*see* **Note 10**).

3.2 Labeling SpoIVA with TCO

1. Slowly thaw frozen aliquots of purified SpoIVA on ice. Centrifuge at 14,000 × g at 4 °C to remove any precipitated protein (*see* **Note 11**).
2. Resuspend stock TCO powder in DMSO to 20 mM and add ten-fold molar excess of TCO reagent directly to SpoIVA. Wrap tube in foil to block light (*see* **Note 12**).
3. Incubate 1–4 h at room temperature or overnight at 4 °C on a shaker.
4. Remove unreacted TCO with Zeba-desalting column equilibrated with 50 mM Tris-HCl, pH 7.5, 400 mM NaCl buffer (*see* **Note 13**).
5. Store SpoIVA-TCO at 4 °C (covered from light) for up to 1 month (*see* **Note 14**).

3.3 Labeling Anti-HER2 Affibody (αHER2) with Methyl-Tetrazine

1. Reduce anti-ERB2 (HER2) affibody with TCEP according to the manufacturer's instructions.
2. Measure the concentration of reduced affibody using UV-spectrophotometer, measuring A_{280} and using the Beer–Lambert law (MW = 6.8 kDa, $\varepsilon = 8480$; *see* **Note 15**).
3. Resuspend stock Methyltretazine-PEG4-malemeide powder in DMSO (*see* **Note 12**) to 20 mM and add ten-fold molar excess directly to the HER2 affibody.
4. Incubate 1–4 h at room temperature on a shaker (*see* **Note 16**).
5. Desalt the methyl-tetrazine-labeled affibody using PD Mini-Trap G-25 columns equilibrated with PBS using manufacturer's instructions.

3.4 SUV Production

1. Transfer 200 μL of (25 mg/mL) DOPC in chloroform into a 16 × 100 mm borosilicate test tube and desiccate overnight at room temperature using speed-vacuum centrifuge (*see* **Note 17**).

2. Hydrate each tube with 1 mL of 0.5× PBS and incubate for 1 h in a water bath set at 42 °C, vortexing vigorously every 15 min.
3. Freeze the sample in an ethanol/dry ice mixture (*see* **Note 18**), then thaw the sample in a water bath set at 42 °C, then vortex vigorously. Repeat five times.
4. Sonicate the sample in a bath sonicator for ~30 min or until the sample becomes transparent (*see* **Note 19**).
5. Transfer lipid solution to microcentrifuge tube and spin down at 13,000 × g for 10 min.
6. Collect supernatant (containing the SUVs) and discard any residual pellet.
7. Repeat steps 5 and 6 for additional purity, as needed (*see* **Note 20**).
8. Store SUVs in glass tube with Teflon-coated cap at 4 °C for up to one week (*see* **Note 21**).

3.5 SSLB Production

1. Centrifuge silica beads (100 μL of 10% w/v stock = 10 mg of beads) 2 min at 13000 × g and remove supernatant.
2. Wash pellet with 1 mL H_2O three times (*see* **Note 22**).
3. Wash pellet with 1 mL Methanol (100%) three times (*see* **Note 22**).
4. Wash pellet with 1 mL NaOH (1 N) three times (*see* **Note 22**).
5. Wash pellet with 1 mL H_2O three times (*see* **Note 22**).
6. Resuspend pellet in 200 μL H_2O (*see* **Note 23**).
7. Add 200 μL of SUVs (5 mg) to 200 μL washed silica beads (10 mg).
8. Incubate for 1 h at room temperature with gentle inversion.
9. Centrifuge sample for 2 min at 13,000 × g and wash three times with 1 mL PBS (*see* **Note 22**).
10. Resuspend SSLB pellet in 1 mL PBS. Final concentration will be 10 mg/mL (~1×10^7 beads/μL).
11. Store SSLBs at 4 °C for up to 1 week (*see* **Note 24**).

3.6 Click Chemistry Reaction

1. Incubate desalted SpoIVA-TCO and Methyltetrazine-αHER2 affibody in a 1:1 ratio.
2. Incubate the reaction for 1 to 4 h at room temperature protected from light (*see* **Note 25**).

3.7 SSHEL Assembly

1. Resuspend SSLB (final concentration 2.5 mg/mL) in SSHEL assembly solution.
2. Wrap samples in foil, incubate overnight at room temperature, using a shaker or inverter.

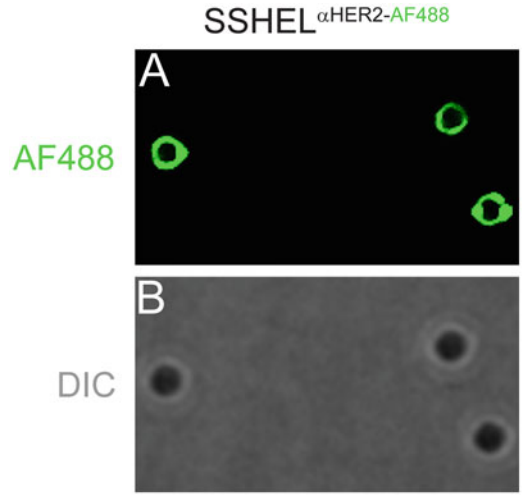

Fig. 2 SSHELs can covalently display anti-HER2 affibodies. SpoIVA on the SSHEL surface were covalently attached to αHER2 affibodies that were labeled with AlexaFluor 488 (AF488). SSHELs were examined using fluorescence microscopy, visualized using (**a**) fluorescence from affibody, or (**b**) differential interference contrast (DIC)

3. The next day, centrifuge the sample and check the concentration of SpoIVA-αHER2 using MESF method or plate reader with a calibration curve if the affibody or the molecule of interest was previously labeled.
4. If using an αHER2 affibody that has been previously labeled with a fluorophore, the distribution of the affibody around SSHEL particles may be optionally observed using fluorescence microscopy (*see* Fig. 2).
5. Labeled SSHELs can be stored indefinitely at −80 °C covered from light.

4 Notes

1. Optional: purchase lyophilized DOPC from Avanti and resuspend in chloroform.
2. Terrific Broth Modified produces a greater protein yield but one may use LB media with 10 g yeast extract, 10 g NaCl, and 10 g Tryptone per liter.
3. One may also purify His_6-tagged SpoVM using Ni^{2+} -NTA agarose as described in reference (8).
4. Pellets can be stored at −80 °C indefinitely.

5. To prevent protein degradation, pre-chill the steel French Press cell and piston at 4 °C prior to use. Collect sample from French Press at ~2 mL/min.
6. Optional: collect 20 μL column flowthrough for analysis by SDS-PAGE.
7. For better yield combine supernatant and Ni^{+2}-NTA agarose in 50 mL tube and rotate on a nutator mixer for 1 h at 4 °C prior to adding to the column.
8. The bed volume of the MonoQ column will dictate the volume of buffer A needed to equilibrate. See manufacturer's instructions.
9. One may use a 10 mL superloop with 1/16″ fitting (130 × 30 mm) (avantor™, Cat# 10497–994).
10. SpoIVA elutes from the MonoQ column at 40% Buffer B for a final buffer composition of 50 mM Tris at pH 7.5, 400 mM NaCl.
11. If noticeable precipitated protein is observed and removed, re-quantify remaining soluble protein using UV-spectrophotometer or Bradford assay.
12. Unused TCO (or Methyltetrazine) in DMSO can be stored for several months at −20 °C protected from light.
13. Optional: quantify SpoIVA-TCO concentration using UV-spectrophotometer or Bradford assay. Expect about a 10% loss of sample.
14. SpoIVA-TCO can be aliquoted and stored indefinity at −80 °C for future use.
15. Expect ≥90% recovery.
16. Optional: shake or rotate reaction overnight at 4 °C.
17. Optional: one may desiccate DOPC in a vacuum centrifuge for 1 h at 45 °C.
18. Optional: one may freeze sample in methanol and dry ice mixture.
19. When using a bath sonicator, fix the 1 mL lipid solution in place just below the surface of the water bath where the top of the water bath is aligned in the middle of the sample. Optional: use a tip sonicator (4 mm titanium tip) for 30 seconds in water–ice bath.
20. Each additional centrifugation should result in reduced observed pellet.
21. When storing SUVs, cap the sample under nitrogen gas and wrap parafilm around vial cap to prevent oxidation. SUVs can be stored for up to a week according to Avanti indications. Do not freeze SUVs.

22. When washing the pellet, use a 200 μL pipet tip to gently pipet up and down to ensure the beads are adequately resuspended and to remove beads adhering to the side of the centrifuge tube. Note: the beads will naturally sediment to the bottom of the centrifuge tube over time.
23. Optional: washed beads can be stored at 4 °C for up to several months in Eppendorf tubes wrapped in parafilm. Pay attention if bacterial contamination occurs.
24. When storing DOPC-coated beads at 4 °C, cap the sample under nitrogen gas and wrap parafilm around vial cap to prevent oxidation.
25. Optional: one may incubate the SpoIVA-TCO and methyltetrazine-αHER2 affibody solution overnight at 4 °C with gentle inverting or mixing protected from light.

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Chapter 18

Structural Determination of Glucosyltransferase C by Cryo-Electron Microscopy

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Abstract

Biofilm formation is a critical factor in the development of cariogenic virulence of *Streptococcus mutans*. *S. mutans* has evolved a concerted mechanism to synthesize a biofilm matrix from dietary sugars by a family of glucosyltransferases (Gtfs). Three Gtfs, GtfB, C, and D utilize sucrose to form a sticky polymer consisting of insoluble and soluble glucans, a biofilm foundation. Each Gtf possesses two distinct domains, an N-terminal enzyme catalytic domain and a C-terminal glucan binding domain. X-ray crystallographic studies have determined a three-dimensional structure of the catalytic domain of GtfC, however, the structure of the C-terminal domain and the overall structure of Gtfs are unknown. Here, we provided a protocol that will guide us to solve Gtf structures using cryo-electron microscopy (cryo-EM). Cryo-EM and X-ray crystallography are two widely used techniques for determining protein structures, and both methods have their own advantages and limitations. Additionally, we also predicted the full-length GtfC structural using AlphaFold2. Overall, the combination of AlphaFold 2 with experimental approaches offers a powerful and synergistic strategy for protein structure determination, accelerating the pace of scientific discovery and enabling new insights into the molecular basis that govern the biosynthetic activities of Gtfs, which may inform the design of more effective treatments for cariogenic biofilms and associated diseases.

Key words Cryo-electron microscopy, Glucosyltransferase, *Streptococcus mutans*, Structural determination, Alpha fold

1 Introduction

Microbiota play key roles in human health and disease [1]. Microbial surface structures are crucial for microbe-host interactions [2, 3]. Bacteria have evolved a host of mechanisms to produce unique surface structures to adapt to their living environments [4, 5]. In the oral cavity, tooth decay-causing organisms represented by *Streptococcus mutans* produce a distinct group of enzymes called glucosyltransferases (Gtf), which are known to specialize in utilizing dietary sugars to create sticky glucans that attach to the tooth surface and produce acidic metabolites that demineralize the tooth surface, resulting in tooth decay [6]. Gtfs have been

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recognized as a molecular glue as they produce glucans by their enzymatic activities, subsequently deposit their bioproduct, sticky glucans to where the bacteria tend to colonize, and then use their own glucans binding modules to secure bacterial interactions with glucans to form a biofilm community connected by a complex matrix network. Gtfs are essential for the development and maturation of this biofilm matrix and are key virulent determinants in the development of *S. mutans*-induced dental caries [7, 8]. However, molecular details that mediate Gtf biochemical activities are not fully understood and it is not clear how two domains of Gtfs coordinate and whether Gtfs interact with each other to shape their functions in the formation of cariogenic biofilm matrix.

There are three Gtfs found in mutans streptococci. GtfB synthesizes predominantly water-insoluble glucans, GtfD synthesizes water-soluble glucans, whereas GtfC makes a mixture of water-soluble and insoluble glucans. Mutants defective in each *gtf* gene exhibit distinct phenotypes, and the loss of individual *gtf* or combined defects in both or all three *gtf* genes exhibit markedly reduced virulence [9, 10]. Other proteins such as VicR, a response regulator of a streptococcal two-component signal transduction system, and BrpA, a biofilm regulatory protein that belongs to a large family of transcriptional factors, also regulate cariogenic biofilm formation and *S. mutans* virulence [11–13], however their precise role in mediating cariogenic biofilms is unknown. Since Gtfs, glucans, and other proteins play a critical role in *S. mutans* virulence, understanding of the molecular details on how Gtfs orchestrate its role in the biofilm network is critical for the development of a novel strategy to prevent bacterial virulence and pathogenesis. We approach this by solving three-dimensional structures of Gtfs by CryoEM. Here, we provide a detailed protocol that outlines our effort in solving CryoEM structures of Gtfs using GtfC as an example.

2 Materials

2.1 Protein Expression

1. *E.coli* BL21 (pET21a-GtfC) [14].
2. LB broth (*see* **Note 1**).
3. LB agar plates containing ampicillin (100 μg/ml) (*see* **Note 1**).
4. Isopropyl ß-D-1-thiogalactopyranoside (IPTG): prepare 1 M stock and sterile-filter with 0.22 μm-syringe filter unit and store at −20 °C.
5. Ampicillin stock: prepare 100 mg/ml and sterile-filter with 0.22 μm-syringe filter unit and store at 4 °C.

6. 14 ml Falcon tubes.
7. Incubator.
8. Shaking incubator.
9. 2-L flasks.

2.2 Protein Purification

1. 250 ml Nalgene centrifuge bottles.
2. Floor centrifuge (e.g., Beckman Coulter J20).
3. Buffer A: 20 mM Tris base, 500 mM NaCl, 35 mM imidazole, pH 8.0 (*see* **Note 2**).
4. Buffer B: 20 mM Tris base, 500 mM NaCl, 500 mM Imidazole, pH 8.0 (*see* **Note 2**).
5. 50 ml conical tubes.
6. Sonicator (e.g., QSONICA sonicators Q500).
7. Nalgene Oak Ridge High-Speed centrifuge tubes.
8. His-trap high-performance column (e.g., Cytiva, 5 mL).
9. FPLC system (e.g., AKTA).
10. 10% SDS-PAGE gels.
11. Amicon Ultra 15 mL filter units (MWCO 50 K).
12. Size exclusion column (GE Superose 6 Increase 10/300 GL).

2.3 Negative Staining

1. GF buffer: 20 mM BisTris, 100 mM NaCl, pH 6.5 (*see* **Note 2**).
2. 1% uranyl acetate (*see* **Note 2**).
3. 300-mesh carbon-coated copper grids (Electron Microscope Sciences, CF-300 Cu).
4. PELCO glow discharger.
5. Transmission electron microscope (TEM) (e.g., FEI Tecnai; *see* Fig. 2a).
6. Self-closing tweezers.
7. Filter papers.

2.4 Frozen Grids

1. Quantifoil R2/2300-mesh copper grids (Electron Microscope Sciences).
2. Vitrobot (Thermo Fisher).
3. Tweezers for Vitrobot.
4. Liquid nitrogen and ethane.
5. Glacios Cryo-TEM.

3 Methods

3.1 Protein Expression

1. Day 1: Streak a LB agar plate, containing ampicillin (100 μg/ml), from the frozen stock of *E.coli* BL21 (pET21a-GtfC) and incubate at 37 °C overnight.
2. Day 2: Inoculate with a single colony from the plate above an 8 ml LB culture, containing ampicillin (100 μg/ml), in a 14 mL Falcon tube. Place the tube on a shaking incubator at 250 rpm and at 37 °C overnight.
3. Day 3: Transfer all the 8 mL overnight inoculum into a 2 L flask containing 1 L of sterile LB broth with ampicillin (100 μg/ml) and incubate at 37 °C with shaking at 250 rpm. When its OD600 reaching to 0.6 (about 2 hours), add 100 μl of 1 M IPTG and place the flask in the shaking incubator at 250 rpm and 18 °C, overnight.

3.2 Protein Purification

1. Day 4: Stop the flask at 18 °C from overnight incubation; pour all the culture contents into 4 x 250 mL Nalgene centrifuge bottles, balance and centrifuge (e.g., Beckman Coulter J20) them at 5000 rpm for 15 min.
2. Decant the supernatant solution; Resuspend all pellets in buffer A in a 50 mL conical tube (Fisher) with a final volume of 30 mL.
3. Lyse the cells and release the proteins by sonication (QSONICA sonicators Q500). Sonicate the 30 mL resuspension for 6 minutes, with each cycle of 5 seconds pulse and 10 seconds pause at 40% amplitude.
4. After sonication, transfer all contents to Nalgene Oak Ridge High-Speed centrifuge tube (Thermo Scientific) and balance with a blank tube with water; Centrifuge (Beckman Coulter J20) them at 17000 rpm for 30 minutes.
5. Carefully transfer the supernatant of cell lysis into a clean 50 mL conical tube; Decant the pellets.
6. Connect Histrap high-performance column (Cytiva, 5 mL) to AKTA prime FPLC and set flow speed at 3 mL/min. Equilibrate the column with 20 mL Buffer A. Load the cell lysis flow through the column and wash the column with 50 mL Buffer A to remove any impurities. Then elute the protein from the column with 15 mL Buffer B.
7. Alternative manual method. Using syringe to inject buffers and samples into the column with the same steps described above.
8. Check the protein purification and purity on the 10% SDS-PAGE gel.

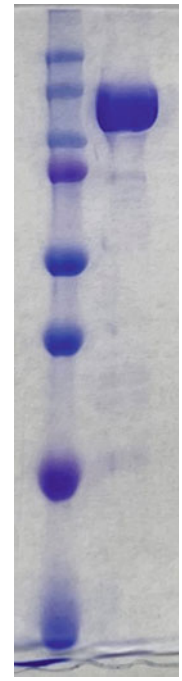

Fig. 1 SDS-PAGE analysis of purified GtfC. Shown are the Bio-Rad precision plus protein markers (lane 1) and purified GtfC (lane 2)

9. Select Amicon Ultra 15 mL (MWCO 50 K) filter unit to concentrate the eluted 15 mL protein solution to ~1 mL at 5000 rpm at room temperature (Eppendorf centrifuge 5804R).
10. Connect size exclusion column (GE Superose 6 Increase 10/300 GL) to AKTA prime. Equilibrate the column with GF buffer at a flow rate of 0.3 mL/min for 30 mL, then inject ~500 μL of the protein into the column and run another 30 mL. Collect fractions after protein injection for 1.5 mL/tube.
11. Run the collected fractions on 10% SDS-PAGE to identify fractions containing GtfC. Select one or two fractions for next step based on both protein intensity and purity revealed by the SDS-PAGE result (*see* Fig. 1).
12. Determine protein concentration of chosen fractions using the following equation:

$$\text{Protein concentration} = \text{OD}_{280} \times \frac{\text{Molecule weight (Dalton)}}{\text{Extinction coefficient (280 nM)}}$$

GtfC molecule weight: 142,914 Da.
GtfC extinction coefficient: 229,660 M^{-1} cm^{-1}.
Molecule weight and extinction coefficient are calculated using SnapGene software based on the GtfC protein sequence.

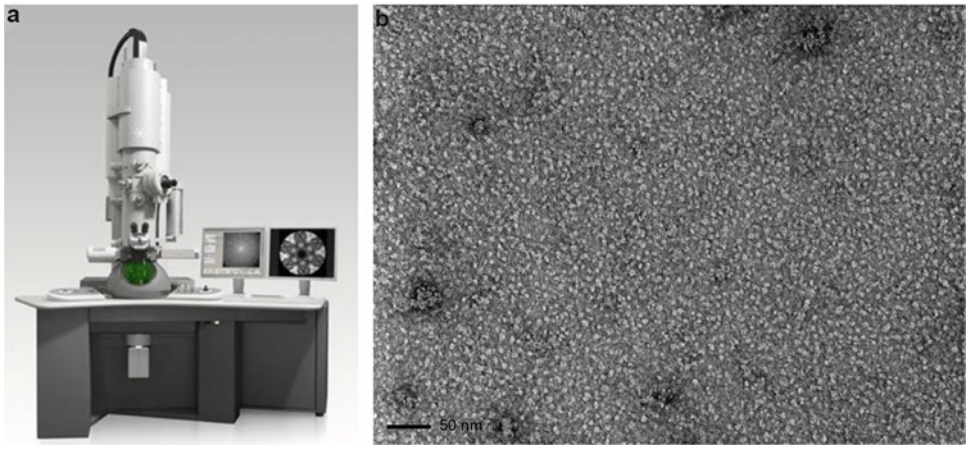

Fig. 2 Electron Microscope & Negative Staining. (**a**) A 120-kV Tecnai transmission electron microscope. (**b**) An example of an EM image using GtfC protein at a concentration of 0.44 mg/mL stained with 1% uranyl acetate

3.3 Electron Microscopy with Negative Staining

The negative stain is a simple method to check the quality of the sample and estimate the adequate concentration for the Cryo-EM.

1. Prepare a serial dilution of GtfC from 0.1 to 1.0 mg/mL in GF buffer. Centrifuge the proteins at 15,800 x g (Eppendorf 5424R) for 10 min at 4 °C to eliminate any possible aggregation.
2. Glow discharge carbon-coated copper grids by placing them in the chamber of PELCO glow discharger, with a glow time of 60 s and an electrical current of 15 mA recommended.
3. Place grids in self-closing tweezers; load a drop about 5 μL of prepared GtfC (0.1 to 1 mg/ml) onto the carbon-coated side of the grid and keep it for 1 to 3 min; remove excess liquid with filter paper on the edge of grids; immediately stain with 1% Uranyl acetate for 30 to 60 s; and remove the excess with filter paper and air-dry.
4. Image the grids under a transmission electron microscope (e.g., FEI Tecnai Transmission Electron Microscope equipped with a 5 megapixel CCD camera; Fig. 2a–b) (*see* **Note 3**).

3.4 Preparation of Cryo-EM Frozen Grids and Screening

After determining protein homogeneity and concentration by negative stain, proceed with freezing grids. This process is often referred to as vitrification.

1. Glow-discharge Quantifoil R2/2300-mesh copper grids with the same parameters as negative staining (**step 2**, Subheading 3.3) (*see* **Note 4**).
2. Fill up the plunger reservoir with deionized water.
3. Turn on Vitrobot (Thermo Fisher). Set the humidity to 100% and temperature to 4 °C. The blotting force and time setting

vary based on the frozen result. For a new protein sample, 4 s blot time and 0 blot force are recommended.

4. Load a pair of filter papers in the Vitrobot and run a blot without protein sample to flat the filter paper.
5. Prepare liquid nitrogen and ethane. Pour liquid nitrogen into cups or small dewars to precool all the tweezers, grid holder, and ethane containers.
6. When the ethane container is cool enough (no bubble and noise from outside liquid nitrogen), fill up ethane to the container, then move the holder to the platform-ring under the Vitrobot.
7. Place the grid on the special designed tweezer for Vitrobot. After click Load Sample on the screen of Vitrobot, the grid will be loaded in the chamber below the filter paper. Then pipette 3 μL of GtfC onto the carbon-coated side of the grid. Click Blot, the filter paper will blot the grid to make a thin aqueous layer.
8. After blotting, the Vitrobot will automatically plunge down the grid into ethane to freeze. Then gently remove the tweezer from Vitrobot and transfer the frozen grid from ethane to liquid nitrogen precooled grid holder. Make sure the frozen grid in ethane or liquid nitrogen all the time.
9. Transfer the grid holder to Clipping station. Place the frozen grid on a precooled O-ring and clip it with C-ring. Then transfer the clipped grid into Cryo grid box and record each grid position. Store the Cryo grid box in Cryo-EM puck or tube in Cryogenic storage tank until data collection.
10. Shut down the Vitrobot and empty the plunger reservoir. Clean up the bench and dry all used containers.
11. Screen grids using a Glacios Cryo-TEM (*see* **Note 5**).

3.5 Data Collection and Processing

1. Once satisfied with the screening results, proceed with cryo-electron microscopy using a Titan high-resolution electron microscope (*see* **Note 6**).
2. After data collection, process the data on a workstation at an EM facility or transfer all the data back to a local computer for processing with CryoSPARC [15], a widely used software suite for single-particle CryoEM data processing.
3. Pre-processing steps:
   (a) Data import. Launch CryoSPARC and create a new project. Import the raw CryoEM data into CryoSPARC. Set up the parameters, such as raw pixel size (Å), accelerating

voltage (kV), spherical aberration (mm), and total exposure dose (e/A^2).

(b) Motion correction. Perform motion correction to align frames within each micrograph using the PatchMotion or MotionCor2 method.

(c) CTF estimation. Estimate the Contrast Transfer Function (CTF) parameters using the CTF estimation module.

4. Particle picking and extraction.

(a) Particle picking. Use the Blob Picker or Template Picker to automatically pick particles from the micrographs. Based on the particle shape and protein size to set the minimum and maximum diameter for particles. Inspect the picked particle to optimize the diameter setting.

(b) Particle extraction. Extract particle images using the selected picking results. Generate a new particle stack for subsequent processing steps.

5. 2D classification: Perform 2D classification to sort particles into different classes based on their structural features. Run several iterations of 2D classification to refine and improve the particle classes. Manually view each of the classes and discard classes with poor quality or contaminations. Usually, all the classes with high resolution (Å) and low Effective Classes Assigned (ECA) should be selected.

6. 3D Reconstruction

(a) Initial model generation – Use an ab initio approach, which is a method that predicts protein structures from their amino acid sequences without using any template or reference structure, to create an initial 3D model with the chosen 2D classes.

(b) 3D refinement – Perform iterative 3D refinement using the selected particles and the initial model. Optimize the refinement parameters to achieve a high-resolution reconstruction. Validate the refinement results using tools like FSC (Fourier Shell Correlation) and map visualization.

7. Post-processing: Apply post-processing techniques, such as particle polishing, local resolution estimation, and mask application to improve the final reconstructed maps (*see* **Note 7**).

8. Model building and visualization: Fit atomic models into the density map using specific pieces of software like Coot [16] or Chimera [17]. Refine and validate the atomic model against the density map (*see* **Note 8**).

4 Notes

1. Use ultra-pure water to prepare media and solutions.
2. Sterile filter using a 0.22 μm membrane filter (e.g., Milipore).
3. The grids can be placed in the original box for long time storage in a clean and dry place.
4. Wear lab coats and goggles.
5. After screening all the frozen grids with different parameters, like protein concentration, blotting time and force, carefully inspect each micrograph from grids with sample coverage and thickness and particle numbers. If the prepared grid is not good enough for data collection, optimize the protein concentration, blotting time, and force to freeze a new grid. If having ice on the grid, watch out the steps when frozen the grid to make sure the grid stay in ethane or liquid nitrogen all the time.
6. Usually, data collection takes about 48 to 72 h to complete. Trained personnel can operate the microscope remotely.
7. GtfC experimental maps: In our experiment, we obtained three initial maps with different parameters (*see* Fig. 3a). The middle one is chosen as it resembles its published crystal structure of GtfC, and used for post processing in cryoSPARC. After refinement in cryoSPARC, two maps were obtained in 3.96/3.76 Å and 3.61/3.63 Å in resolution (*see* Fig. 3b), separately.
8. We used GtfC structure (PDB ID, 3AIE) solved by x-ray crystallography [18] as a structure model to manually fit into our experimental map using UCSF Chimera (*see* Fig. 4). Our CryoEM structure of GtfC is highly similar to its published crystal structure of GtfC. Nonetheless, both structures only display the catalytic domain, while missing the glucan binding domain.

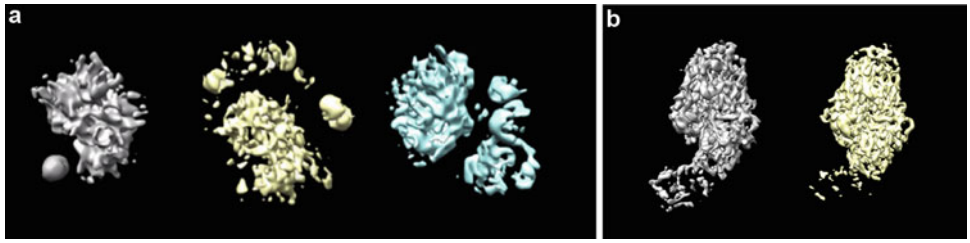

Fig. 3 The CryoEM maps of GtfC. (**a**) Three initial maps were generated using cryoSPARC. (**b**) The GtfC maps after post-processing and refinement

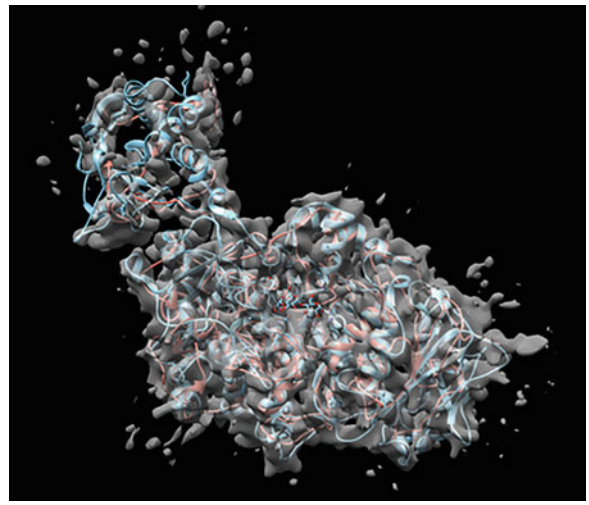

Fig. 4 The structure of GtfC fitting in CryoEM map. Cryo-EM map is in gray, GtfC crystal structure highlighted by the colored ribbon

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