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Chapter 9.

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Summary.

Inner membrane proteins are inserted into the membrane via α-helices. These helices are not only membrane anchors but usually mediate specific interactions with membrane protein partners or participate to energetic processes. The number, location and orientation of these helices is referred as topology. Bitopic membrane proteins that consist of a single membrane-embedded domain connecting two soluble domains are distinguished from polytopic ones that consist to multiple-spanning TMH connected by extramembrane domains. Defining inner membrane protein topology could be achieved by different methods. Here, we describe a protease accessibility assay that allows to define topology based on digestion profiles.

1. Introduction

Bacterial secretion systems are multiprotein machines that catalyze the traffic of protein substrates across the cell envelope (***1***). Most secretion systems described so far assemble large channels constituted of inner and outer membrane proteins (***1***). In secretion systems, inner membrane proteins are crucial to assemble platforms for pilus polymerization, substrate recruitment and selection or for energetic purposes (***1***). Defining inner membrane protein topology, a term referring to the number, position and orientation of transmembrane helices (TMH), is therefore an important step to characterize these proteins. Depending on the number and position of these TMH, inner membrane proteins are categorized into bitopic and polytopic proteins (Figure 1). Bitopic membrane proteins consist of a single membrane-embedded domain connecting two soluble domains located in two different compartments. The TMH of bitopic proteins could be located at the N- or C-terminus. By contrast, polytopic membrane proteins consist to multiple TMH that are connected by extramembrane domains,

called loops. Bitopic proteins with N-terminal TMH are relatively common and this category includes GcpC, YscD and VirB10, subunits associated with the Type II (T2SS), Type III (T3SS) and Type IV (T4SS) secretion systems (***2-4***). Bitopic proteins with C-terminal TMH, also called C-tail proteins, are scarce. In secretion systems, only the Type VI secretion system (T6SS) TssL protein has been demonstrated to adopt this topology (***5***, ***6***). Polytopic membrane proteins are also commonly associated with secretion system, and this category includes the HlyB, YscU, VirB6 and TssM proteins that are respectively associated with T1-, T3-, T4- and T6SS (***7-11***).

Inner membrane protein TMH position and orientation could be predicted using computational methods, based on hydrophobicity patterns and the "positive-inside" rule (*see* Chapter XX by Nielsen). Several approaches have also been developed to experimentally define protein topology (***12***, ***13***), including the *pho-lac* dual reporter system (*see* Chapter XX by Karimova) and the substituted cysteine accessibility method (SCAM, *see* Chapter XX by Bogdanov). In this chapter, we will describe a third approach, based on the accessibility of extramembrane, soluble domains to exogenous proteases. In addition to assessing inner membrane topology, protease accessibility assays are also of interest to test *in vitro* translocation of proteins (***6***, ***14***) and to test whether proteins are subjected to conformational changes *in vivo* (***15-17***, *see* Chapter XX by Larsen).

2. Material

2.1. Cell growth and spheroplast preparation.

1. Lysogeny Broth (LB) or the recommended medium to grow the strain of interest.
2. TNS buffer: 20 mM Tris-HCl, pH8.0, 100 mM NaCl, 30% sucrose: Dissolve 0.243 g of Tris(hydroxymethyl) aminomethane, 0.684 g of NaCl and 30 g of sucrose in sterile distilled water (final volume of 100 mL). Adjust the pH to 8.0 with 1 M HCl.

3. TN buffer: 20 mM Tris-HCl, pH8.0, 100 mM NaCl: Dissolve 0.243 g of Tris(hydroxymethyl) aminomethane and 0.684 g of NaCl in 100 mL of sterile distilled water. Adjust the pH to 8.0 with 1 M HCl.
4. 0.5 M EDTA, pH8.0: Dissolve 1.86 g of Ethylene diamine tetraacetic acid (EDTA, disodium salt) in 10 mL of sterile distilled water. Adjust the pH to 8.0 with 10 M NaOH.
5. Lysozyme stock solution (100×). 10 mg/mL Lysozyme: Dissolve 10 mg of goose egg lysozyme in 1 mL of sterile distilled water. Store at -20°C.
6. Incubator.
7. Spectrophotometer to measure bacterial density.
8. Labtop centrifuge.

2.2. Protease accessibility assay.

1. Triton X-100 stock solution. 10% Triton X-100: Mix 1 mL of 100% Triton X-100 with 9 mL of sterile distilled water (*see* **Note 1**). Store at room temperature.
2. Carboxypeptidase Y stock solution (100×). 10 mg/mL Carboxypeptidase Y: Dissolve 10 mg of purified carboxypeptidase Y in 1 mL of sterile distilled water. Store at -20°C.
3. Proteinase K stock solution (100×). 10 mg/mL Proteinase K: Dissolve 10 mg of purified proteinase K in 1 mL of sterile distilled water. Store at -20°C.
4. Cocktail of protease inhibitors (Complete, Roche or equivalent).
5. Phenylmethylsulfonyl fluoride (PMSF) stock solution (100×). 100 mM PMSF: Dissolve 17.4 mg of PMSF in 1 mL of absolute ethanol (*see* **Note 2**). Store at -20°C.
6. 50% TCA solution: Dissolve 50 g of trichloroacetic acid (TCA) in 30 mL of distilled water. Complete to 100 mL with distilled water (*see* **Note 3**).
7. Acetone.
8. Vortex.

2.3. Sample analysis by SDS-PAGE and immunodetection.

1. SDS-PAGE loading buffer: 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue.
2. Water bath at 96°C.
3. Mini-gel caster system and SDS-PAGE apparatus.
4. Protein blotting apparatus
5. Antibodies for protein immunodetection.

3. Method

3.1. Cell growth and spheroplast preparation (*see* **Note 4**).

1. Grow a 30-mL culture in the appropriate medium to allow cell growth and the production of the protein of interest (*see* **Note 5**).
2. Collect cells by centrifugation at 5,000 × g for 10 minutes at 4°C.
3. Discard the supernatant and gently resuspend the cell pellet in to optical density at 600 nm (OD_{600}) of 12 in ice-cold TNS buffer. Incubate on ice for 10 minutes.
4. Add EDTA at 1 mM final concentration (*see* **Note 6**). Incubate on ice for 5 minutes.
5. Add Lysozyme at the final concentration of 100 µg/mL and incubate on ice for 15-40 minutes (*see* **Note 7**).
6. Dilute the sample twice with ice-cold TN buffer, mix by gently inverting the tube and keep on ice for 10 minutes.
7. Collect spheroplasts by centrifugation at 10,000 × g for 5 minutes at 4°C.
8. Gently resuspend spheroplasts to an OD_{600} of 6 in ice-cold TN buffer.

3.2. Protease accessibility (*see* **Notes 8** and **9**).

1. Divide the cell suspension in 5 samples, numbered 1-5. Sample 1 will remain untreated.

2. Add 1% (final concentration) of Triton X-100 in samples 3 and 5 to lyse spheroplasts (*see* **Note 9**). Mix by vortexing and incubation 10 minutes on ice.

3. Add Carboxypeptidase Y (100 µg/mL final concentration from the 10 mg/mL stock solution) in tubes 2 and 3. Incubate for 30 minutes on ice.

4. Add Proteinase K (100 µg/mL final concentration from the 10 mg/mL stock solution) in tubes 4 and 5. Incubate for 30 minutes on ice.

5. Quench the proteolysis reaction by adding PMSF and inhibitor cocktail in tubes 1-5. Incubate for 5 minutes on ice.

6. Add 0.5 volume of 50% TCA in tubes 1-5. Mix by vortexing and incubate for 20 minutes on ice.

7. Collect precipitated material by centrifugation at 20,000 × g for 20 minutes at 4°C.

8. Discard supernatant and add 500 µL of acetone. Vortex.

9. Collect precipitated proteins by centrifugation at 20,000 × g for 20 minutes at 4°C.

10. Discard supernatant and keep tubes open until the pellet is dry (*see* **Note 10**).

3.3. Sample analysis by SDS-PAGE and immunodetection.

1. Resuspend the pellet in SDS-PAGE loading buffer by throughout vortexing.

2. Boil the samples in a water bath for 5-10 minutes (*see* **Note 11**).

3. Proceed to SDS-PAGE and immunoblotting using your favorite protocol.

A schematical example of expected results for topology mapping using proteolysis is shown in Fig. 2.

4. Notes

1. Triton X-100 is a detergent used to lyse cells and solubilize a subset of membrane proteins. It is a viscous solution and therefore should be pipetted slowly and with care.

2. PMSF is a serine protease inhibitor with a short half-life. Due to its instability in solution, it is recommended to prepare fresh solution extemporarily.

3. Trichloroacetic acid is highly irritating. It should be therefore manipulated with care (gloves, laboratory suit and glasses).

4. For Gram-negative bacteria, spheroplasts should be prepared to provide access of the protease to the periplasmic side of the inner membrane. For Gram-positive bacteria, grow, harvest and resuspend the cells as specified as in steps 1-2 of section 3.1 and then proceed to step 8 of section 3.1.

5. Use the appropriate medium to grow the cells. In case the expression of the gene coding the protein of interest needs to be induced, add the inducer at the appropriate concentration.

6. This concentration of EDTA is commonly used for disturbing the lipopolysaccharide layer of the outer membrane in *E. coli* cells. Other bacterial strains may need higher concentrations of EDTA.

7. Lysozyme concentration and incubation time should be adapted to the bacterial strain used in the assay. Efficient spheroplast preparation of most Gram-negative bacteria requires incubation on ice for 15-40 minutes.

8. Protease accessibility should be tested with two proteases: one processive exopeptidase hydrolizing from the C-terminus of the protein (*e.g.*, carboxypeptidase Y) and one endopeptidase with low or broad specificity (*e.g.*, trypsin, papain, proteinase K). When using the calcium-dependent proteinase K, add 0.1 mM $CaCl_2$ in the TN buffer. For

simplification purposes, this protocol describes an assay with carboxypeptidase Y and proteinase K.

9. Appropriate controls include protease accessibility assays with lysed spheroplasts. Spheroplasts are lysed by the addition of 1% Triton X-100. The presence of Triton X-100 in the assay buffer does not interfere with most proteases.

10. If available, the pellet could be dried using a vacuum SpeedVac concentrator (or equivalent).

11. A number of highly hydrophobic polytopic inner membrane proteins precipitate in SDS-PAGE loading buffer when boiled. For the first assay, keep the concentrating gel during the immunoblot to verify that the protein is not retained in the well.

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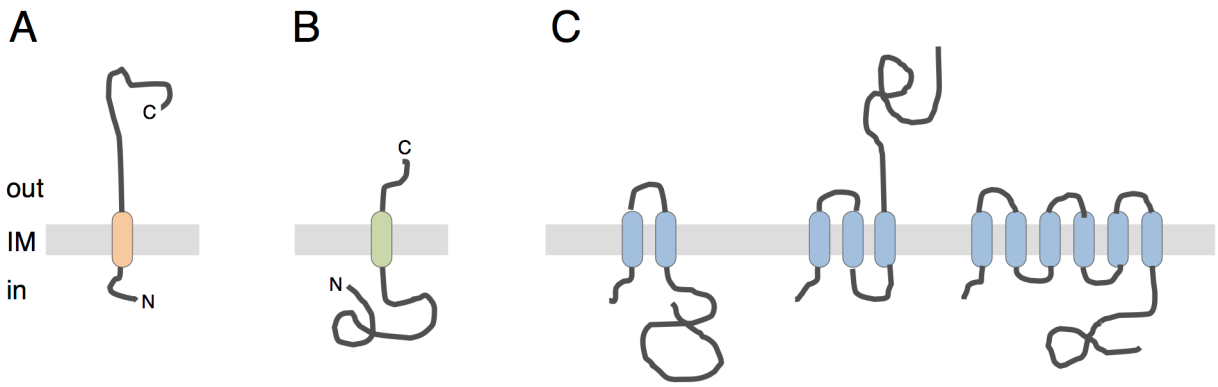
Legend to Figures

Figure 1. Nomenclature of inner membrane proteins with selected topologies. Are shown the topologies of a bitopic protein with a N-terminal TMH (A), a bitopic protein with a C-terminal TMH (B) and polytopic proteins with different numbers of TMH (C). Representative examples of inner membrane (IM) proteins with these topologies associated to bacterial secretion systems are listed below. For polytopic proteins, the number of trans-membrane segments are indicated in brackets.

Figure 2. Schematic representation of expected results. The expected immuno-blot results for inner membrane proteins with the topology shown below are schematically represented. Samples 1-5 are shown (1, untreated sample; 2, Carboxypeptidase Y; 3, Carboxypeptidase Y on Triton X-100-lysed spheroplasts; 4, Proteinase K; 5, Proteinase K on Triton X-100-lysed spheroplasts). The representation of the protein degradation products corresponding to the immuno-detected bands are shown on right of each "blot".



bitopic protein
N-terminal TMH

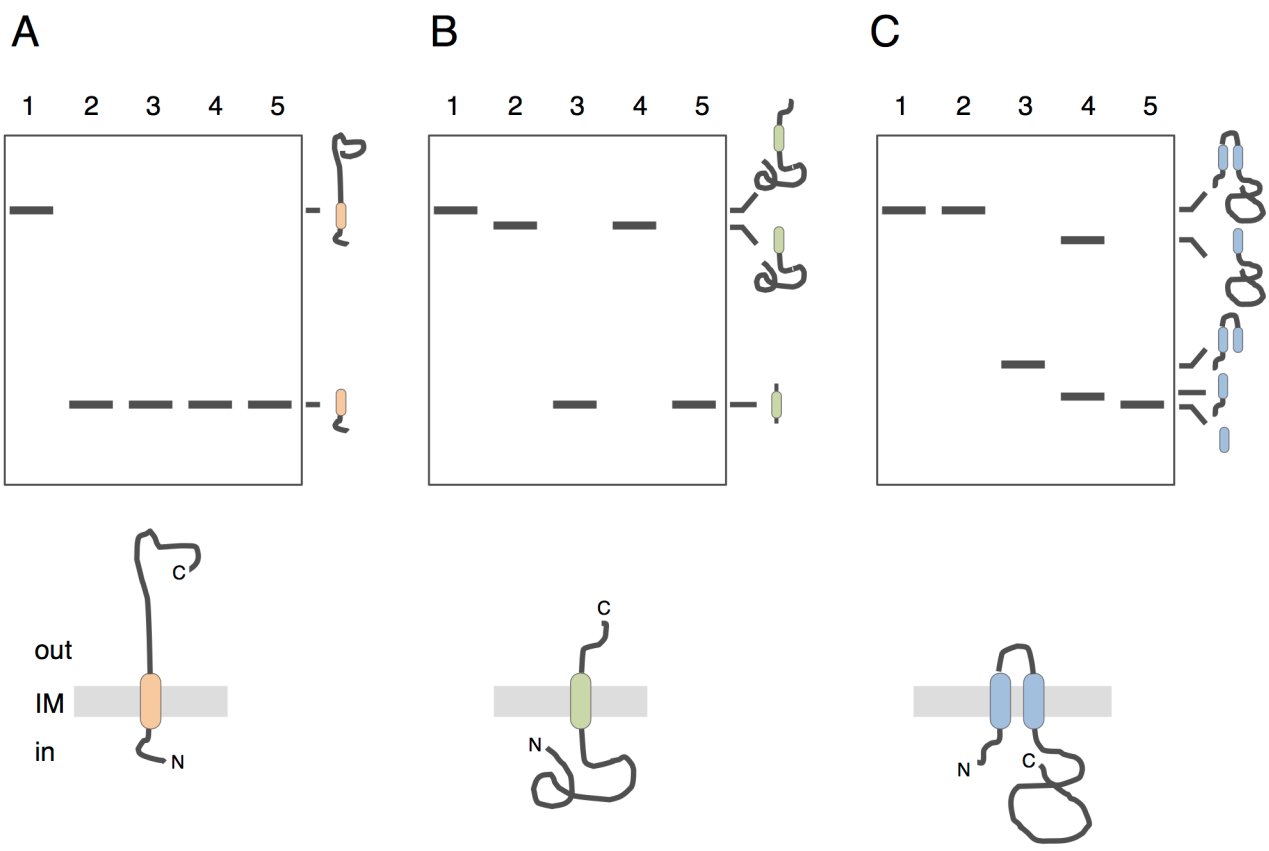
T2SS: GspC
T3SS: YscD
T4SS: VirB8, VirB10
T9SS: PorM

bitopic protein
C-terminal TMH

T6SS: TssL

polytopic proteins

T1SS: HlyB (6)
T3SS: YscU (4)
T4SS: VirB6 (5), VirD4 (2)
T6SS: TssM (3), TagL (3)
T9SS: PorL (2)


A
B
C
1 2 3 4 5
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1 2 3 4 5
C
out
IM
in
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C
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