Identification of Effectors: Precipitation of Supernatant Material

Nicolas Flaugnatti, Laure Journet

Chapter 30

Identification of effectors: Precipitation of supernatant material

Running Head: TCA precipitation

Nicolas Flaugnatti and Laure Journet*

Laboratoire d'Ingénierie des Systèmes Macromoléculaires, UMR7255, Institut de Microbiologie de la Méditerranée, Aix-Marseille Univ – CNRS, Marseille, France

*Correspondence:

Laure Journet

LISM - UMR7255

Institut de Microbiologie de la Méditerranée

Aix-Marseille Univ - CNRS

31 Chemin Joseph Aiguier

13402 Marseille Cedex 20

France

ljournet@imm.cnrs.fr

i. Summary

Bacterial secretion systems allow the transport of proteins, called effectors, as well as external machine components in the extracellular medium or directly into target cells. Comparison of the secretome, *i.e.* the proteins released in the culture medium, of wild-type and mutant cells provide information on the secretion profile. In addition, mass spectrometry analyses of the culture supernatant of bacteria grown in liquid culture under secreting conditions allows the identification of secretion systems substrates. Upon identification of the substrates, the secretion profile serves as a tool to test the functionality of secretion systems. Here we present a classical method used to concentrate the culture supernatant, based on TCA precipitation.

ii. Key words: supernatant, TCA precipitation, secretome

1. Introduction

Bacterial secretion systems are macromolecular machines dedicated to the transport of proteins across the cell envelope. These secretion systems deliver effectors outside the cell, either in the medium (T1SS, T2SS, T5SS, T9SS) or directly into target cells (T3SS, T4SS, T6SS) ***(1)***. Secretion of effector proteins into the milieu can be observed in these systems and the analysis of secretion supernatant has been widely used either to identify new secreted effectors or to probe the functionality of secretion systems. For contact-dependent system such as the T3SS, in vitro secretion in the medium can be observed upon certain conditions (such as Ca^{2+} depletion, acidic pH, etc.) ***(2, 3)***. If effectors can be predicted by bioinformatics approaches for several of these secretion systems (*see* **Chapter 2**), it is not always possible, and analysis of the content of the culture media, the so-called secretome, using global proteomic approaches has been widely used to identify secretion system substrates in T2SS ***(4-7)***, T6SS ***(8-10)***, T3SS ***(11)*** or T9SS ***(12)***.

Upon identification of the substrates, the secretion profile is used to test the functionality of the secretion system using SDS-PAGE of supernatant fraction followed by Coomassie blue staining or immuno-staining by Western blot detection of specific effectors or components of the machinery. In some secretion system, such as T3SS or T6SS, structural external components are released in the milieu upon secretion and can also be used to test the proper assembly of the system. For example, the Hcp release assay is widely used to probe the functionality of the T6SS (*see* also **Chapter 32**).

Such analyses of secretomes require to concentrate the dilute solutions that are the culture supernatant or the biological fluids. This can be achieved using trichloroacetic acid (TCA) precipitation and acetone-based protocols ***(13,14)***. Alternative protocols have been proposed, using acetone alone, methanol/chloroform ***(15)***, or a combination of pyrogallol red, molybdate and methanol ***(16)***.

Here we detail the most classical assay used to precipitate proteins of bacterial culture supernatant based on TCA precipitation that is used thoroughly in secretion systems studies. First, cells and supernatant are

separated by centrifugation. Cell-free culture supernatant fraction samples are then obtained by further centrifugation and filtration and subjected to TCA precipitation before analysis by mass spectrometry or Western blot.

2. Materials

1. Lysogeny Broth (LB) or the recommended medium to grow the strain of interest in secreting conditions.
2. Trichloroacetic Acid (CCl_3COOH, MW: 163.39, TCA): 100% (w/v) **.** Add 227 mL of ultrapure water to a previously unopened bottle containing 500 g of TCA (*see* **Note 1**). Wear personal protective equipment and work under a fume hood.
3. Sodium Deoxycholate (DOC): 16 mg/mL (Optional, *see* **Note 2**). Store at room temperature.
4. Acetone. Pre-chill before use.
5. Refrigerated centrifuge capable of 21,460 × g or table top centrifuge (*see* **Note 3**).
6. 0.22-μm-pore-size syringe filters (*see* **Note 4**).
7. 2-mL syringe.
8. 3 M Tris-HCl, pH 8.8
9. SDS-PAGE loading buffer: 60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue.
10. Boiling water bath or thermomixer.
11. Vortexer
12. 2-mL microtubes (Safe-lock) (*see* **Note 3**)
13. Fume hood and personal protective equipment for TCA handling.
14. Spectrophotometer to measure absorbance at λ=600 nm
15. SDS-PAGE and Protein transfer apparatus

3. Methods

1. Grow a 10 mL bacterial strain culture in the appropriate medium and conditions allowing secretion (*see* **Note 5** and **Note 6)**. Measure the optical density at λ=600 nm (OD_{600}).

2. Dispose the culture in 2-mL microtubes (*see* **Note 7)** and pellet cells by centrifugation at 6,000 × g for 5 min.

3. Carefully remove 1.8 mL of supernatant and transfer it in a new microtube and keep it on ice before performing step 5.

4. Carefully discard the remaining 200 µL of supernatant from the cell pellet obtained in step 3. (Centrifuge again at 6,000 × g for 5 min if the cells from the cell pellet started to resuspend). Keep this total cell fraction pellet on ice before resuspending the pellet in an appropriate volume of SDS-PAGE loading buffer (The equivalent of 0.2-0.5 OD_{600} units (ODU) / 10 µL). Store on ice (or -20°C).

5. Centrifuge the 1.8 mL supernatant fraction obtained in step 3 at 16,000 × g at 4°C for 5 min. Recover carefully the supernatant and transfer it in a new microtube. Avoid to recover the remaining cells from the pellet, if any.

6. Filter-sterilize the supernatant using a syringe 0.22-µm filter and transfer the filtered supernatant directly in a new microtube. Check the volume (around 1.5 mL). This fraction constitutes the cell free fraction (*see* **Note 8**).

7. Add TCA to a final concentration of 20% (add 375 µL of TCA to 1.5 mL of filtered supernatant). Invert 4 times to mix, vortex and keep on ice for one hour to overnight.

8. Centrifuge at 21,000 × g for 30 min at 4°C.

9. Discard the supernatant as much as possible (*see* **Note 9**).

10. Resuspend the pellet in 400-500 µL of cold acetone. Vortex.

11. Centrifuge at 21,000 × g for 15 min at 4°C. Discard the supernatant with a pipet and further on a paper towel. Leave the tube open at room temperature to dry the pellet (*see* **Note 10**).

12. Resuspend the pellet in appropriate buffer for further analysis (such as mass spectrometry) or go to step 13 for SDS-PAGE analysis.

13. Resuspend TCA-precipitated pellets of supernatant fractions in an appropriate volume of SDS-PAGE loading buffer (1 ODU / 10 µL). If the TCA-precipitated sample turns yellow, add 1 µl (or more) of Tris-HCl, pH8.8.

14. Vortex. Heat the samples from step 4 (whole cell fraction) and step 13 (cell free supernatant precipitated fraction) at 95°C for 10 min. (*see* **Note 11**).

15. Analyse whole cells samples and cell free supernatants by SDS-PAGE, followed by Coomassie blue staining or immunoblot. If performing Western blot, include a control for cell lysis, using antibodies detecting an internal protein. Alternatively, check the Commassie or Silver staining profile.

4. Notes

1. For a safe and easy preparation, avoid weighting out the TCA crystalline powder, as it becomes easily syrupy upon contact with air moisture. The TCA solution must be kept in dark glass bottle. It is very corrosive and should be handle with care with suitable protection. Do not use plastic containers.
2. DOC may be used as a carrier to assist protein precipitation. If using DOC, add the DOC stock solution at the final concentration of 0.16 mg/mL to the cell free fraction obtained in step 6, vortex and leave on ice for 30 min, then proceed to TCA precipitation as described in step 7. DOC should be washed out with further acetone washing steps (repeat steps 10-11 three times). However, it could be a problem with further mass spectrometry analysis.
3. Table top centrifuge at maximum speed may be sufficient, however we generally use higher speed. TCA resistant tubes should be use, such as Eppendorf tubes (check with your manufacturer for tube compatibility).

4. In principle, any 0.22 µm filter may be used. However, we had experience with a secreted protein that was retained on PVDF filters, so we moved to Polyether sulfone (PES) filters. Be aware that material of the filter may be of importance.
5. A "non secreting strain" should be used as a control, such as a mutant in a core component, or the ATPase energizing the assembly of the secretion machinery or the substrate transport.
6. You must find conditions where secretion can be detected in vitro. As effectors can be secreted in low levels, high sensitivity mass spectrometry methods may be required ***(10)***. In case the secretion system is not produced in laboratory conditions, native endogenous promoter(s) may be swap by an inducible promoter (P*tac*, P*lac*, P*BAD*, etc.) to artificially induce the expression of the secretion system ***(17)***.
7. A 5-10 mL of culture is generally sufficient. We usually transfer 2 mL of supernatant in 2-mL microtubes, leading to the recovery of 1.5 mL of cell free supernatant. An equivalent of 1 OD_{600} unit will be loaded on the gel for supernatant fractions analysis. For scale up experiments, you will have to use tubes with bigger volume compatible with high speed spin and that are resistant to TCA. Appropriate 50-mL tubes (polyether) may be used, check first with your manufacturer for TCA compatibility.
8. At this stage, for bacteria producing high levels of vesicles (*e.g* for T9SS in Bacteroidetes), an additional ultracentrifugation (30,000 × g for 4 h at 4°C) will allow to separate vesicles from vesicle-free supernatant ***(12)***.
9. Check the orientation of the microtube before the centrifugation step, as the pellet is not always visible at this stage.
10. You may use a vacuum concentrator (SpeedVac or equivalent) for 10 min to evaporate the acetone. However, pellets may be more difficult to resuspend if too dry, and this step may decrease recovery of the samples.
11. In some cases we observed that an additional freezing at -20°C in SDS-PAGE loading buffer helps resuspension of TCA precipitates.

Acknowledgments

This work was supported by the Centre National de la Recherche Scientifique, the Aix-Marseille Université and grants from the Agence Nationale de la Recherche (ANR-14-CE14-0006-02 and ANR-15-CE11-0019-01). Ph.D studies of N.F is supported by the ANR-14-CE14-0006-02 grant.

References

1. Costa, T.R., Felisberto-Rodrigues, C., Meir, A., Prevost, M.S., Redzej, A., Trokter, M., Waksman, G. (2015) Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat Rev Microbiol.* 13, 343-59.
2. Cornelis, G.R, Biot, T., Lambert de Rouvroit, C., Michiels, T., Mulder B., Sluiters, C., Sory, M.P., Van Bouchaute, M., and Vanooteghem, J.C. (1989). The Yersinia yop regulon. *Mol. Microbiol*. 3, 1455-1459.
3. Beuzon, C.R., Banks, G., Deiwick, J., Hensel, M. and Holden, D.W. 774. (1999) pH-dependent secretion of SseB, a product of the SPI-2 type III secretion system of Salmonella typhimurium. *Mol. Microbiol.* 33, 806-816.
4. Coulthurst, S.J, Lilley, K.S, Hedley, P.E, Liu, H, Toth, I.K and Salmond, G.P. (2008) DsbA plays a critical and multifaceted role in the production of secreted virulence factors by the phytopathogen Erwinia carotovora subsp. atroseptica. *J Biol Chem* 283, 23739-53.
5. Kazemi-Pour, N., Condemine, G. and Hugouvieux-Cotte-Pattat, N. (2004) The secretome of the plant pathogenic bacterium Erwinia chrysanthemi. *Proteomics* 4, 3177-86.
6. Sikora, A.E, Zielke, R.A., Lawrence, D.A, Andrews, P.C, Sandkvist, M. (2011) Proteomic analysi is of the Vibrio cholerae type II secretome reveals new proteins, including three related serine proteases. *J Biol Chem* 286,16555-66

7. Burtnick, M.N., Brett, P.J. and DeShazer, D. (2014) Proteomic analysis of the Burkholderia pseudomallei type II secretome reveals hydrolytic enzymes, novel proteins, and the deubiquitinase TssM. *Infect Immun* 82, 3214-26.
8. Hood, R.D., Singh, P., Hsu, F., Güvener, T., Carl, M.A., Trinidad, R.R., Silverman, J.M., Ohlson, B.B., Hicks, K.G., Plemel, R.L., Li, M., Schwarz, S., Wang, W.Y., Merz, A.J., Goodlett, D.R. and Mougous, J.D. (2010) A type VI secretion system of Pseudomonas aeruginosa targets a toxin to bacteria. *Cell Host Microbe.* 7, 25-37
9. Russell, A.B., Singh, P., Brittnacher, M., Bui, N.K., Hood, R.D., Carl, M.A., Agnello, D.M., Schwarz, S., Goodlett, D.R., Vollmer, W. and Mougous, J.D. (2012) A widespread bacterial type VI secretion effector superfamily identified using a heuristic approach. *Cell Host Microbe* 11, 538-49
10. Fritsch, M.J., Trunk, K., Diniz, J.A., Guo, M., Trost, M. and Coulthurst, S.J. (2013) Proteomic identification of novel secreted antibacterial toxins of the Serratia marcescens type VI secretion system. *Mol Cell Proteomics* 12, 2735-49.
11. Deng, W., de Hoog, C.L., Yu, H.B., Li, Y., Croxen, M.A., Thomas, N.A., Puente, J.L., Foster, L.J., and Finlay, B.B. (2010) A comprehensive proteomic analysis of the type III secretome of Citrobacter rodentium *J Biol Chem.* 285, 6790-800.
12. Veith, P.D., Chen, Y.Y., Gorasia, D.G., Chen, D., Glew, M.D., O'Brien-Simpson, N.M., Cecil, J.D., Holden, J.A., Reynolds, E.C. (2014) Porphyromonas gingivalis outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors. *J Proteome Res.* 13, 2420-32.
13. Hwang, B. J. and Chu, G. (1996) Trichloroacetic acid precipitation by ultracentrifugation to concentrate dilute protein in viscous solution. *BioTechniques* 20, 982-984.
14. Ozols J. (1990) Amino acid analysis. *Methods Enzymol* 182, 587-601
15. Wessel, D. and Flügge, U.I. (1984) A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem* 138, 141-3.

16. Caldwell, R.B and Lattemann, C.T. (2004) Simple and Reliable Method To Precipitate Proteins from Bacterial Culture Supernatant. *Appl Environ Microbiol* 70, 610–612

17. Gueguen, E., and Cascales, E. (2013) Promoter swapping unveils the role of the Citrobacter rodentium CTS1 type VI secretion system in interbacterial competition. *Appl Environ Microbiol.* 79, 32-8.