PNEUMOCOCCAL CELL WALL PURIFICATION

Charles Yin and Jason Fan: **August 8, 2012**
Bowdish Lab, McMaster University
Hamilton, ON, Canada
www.bowdish.ca

BACKGROUND

- The bacterial cell wall is a fascinating structure that serves as both a protective shield for invasive pathogens and as a means of bacterial recognition by the host innate immune system. For many applications it will be desirable to obtain purified cell wall.
- Although designed for purification of pneumococcal cell wall, this protocol can be used (with some modifications) for the purification of cell walls from other bacteria, both Gram-positive and negative.
- This protocol was adapted from Tuomanen et al.[1] and Bui et al.[2]

NOTES

- This protocol is designed for the purification of crude cell wall containing teichoic acids, further steps should be taken if teichoic acids are to be removed.
- The volume used herein is 100ml of initial bacterial culture, larger or smaller volumes can be used. The volume used should be dependent on the downstream application of cell wall. For the signaling assays done in our lab, 100ml of culture was found to be sufficient.
- Note that for 1L of bacteria it takes ~8-10h of growth time starting from the same stock as described below.

EQUIPMENT

- Equipment:
  - Tissue homogenizer capable of handling bacteria (we use the NextAdvance BulletBlender)
  - Spectrophotometer
  - Hotplate or heating block
  - Level II biosafety cabinet for handling live *S. pneumoniae*
- Materials:
  - RNase, DNase and proteinase K
  - 1M Tris-HCl (pH 7.0 and pH 8.0)
  - 8M LiCl
  - 1M EDTA
  - 1M NaCl
  - acetone
  - SDS (5% and 2%)
  - $MgSO_4$ and $CaCl_2$ (solid)
  - 0.1% crystal violet

PROTOCOL

Bacterial growth and collection:

- Preparatory Work:
    - Prepare 100ml of sterile TSB
    - Have ready 1ml stock of OD_{600} = 0.15 *S. pneumoniae*
    - Always work with live bacteria in a biosafety cabinet

1. Add entire bacteria stock to 100ml of TSB.
2. Incubate stationary at 37°C for ~5h until OD_{600} = 0.5. Check with a spectrophotometer every 30min starting at the 4h mark.
3. Before the end of the incubation period, place some 50mM Tris-HCl (pH 7.0) on ice.
4. Pellet bacteria by centrifugation at 4,000rpm for 10min at 4°C.
5. Dispose of supernatant. Resuspend pellet in 5ml ice-cold Tris-HCl. Keep on ice.
6. Heat 15ml of 5% SDS to 95°C.
7. Add resuspended *S. pneumoniae* to hot SDS dropwise and incubate at 95°C for 30min. **This is best done with a hot plate inside the biosafety cabinet!**
8. Centrifuge at 4,000rpm for 10min at room temperature.
9. Wash 2× with 1M NaCl and repeatedly with dH_2O until no traces of SDS can be detected.
    - Presence of contaminating SDS can be assayed using a methylene green stain, detailed in **Detection of contaminating SDS using methylene green**.
10. Resuspend cells in 2 pellet volumes of dH_2O.

- Concluding Work:
    - Can be stored at 4°C at this point if desired.
    - The purpose of the SDS extraction outlined above is to kill the *S. pneumoniae* while inactivating its autolysins. This process need not be done for bacteria that do not possess autolysins.

Removal of proteins and nucleic acids:

- Preparatory Work:
    - Prepare 1ml of 100mM Tris-HCl (pH 8.0) with 20mM $MgSO_4$
    - Have a heating block at 37°C.

1. Aliquot cell wall prep into a 5ml BulletBlender tube. At an amount of 0.5mm beads equal to volume of pellet.
2. Place tube into blender along with two tubes with just beads. **Space evenly!**
3. Homogenize at speed 8 for 3min.
4. Aliquot homogenized mix into a fresh 1.5ml tube.
5. Centrifuge at 3,000rpm for 10min. Remove supernatant to a fresh tube. Discard pellet.
6. Centrifuge supernatant at 10,000rpm for 30min. Remove and discard supernatant.
7. Resuspend pellet in 750µl 100mM Tris-HCl (pH 8.0) containing 20mM $MgSO_4$.
8. Add 10µg/ml DNase A and 50µg/ml RNase I. Incubate at 37°C for 2h.
9. Add 100µg/ml proteinase K and 10mM worth of $CaCl_{2(s)}$. Incubate at 37°C overnight.
10. Concluding Work:
    - DNA, RNA and proteins are removed at this stage.

Cell wall purification

- Preparatory Work:

    - Have heating blocks at 95°C and 37°C (or turn down the temperature of the heating block after Step 1)

1. Add 750µl 2% SDS. Incubate at 95°C for 15min.
2. Centrifuge at 10,000rpm for 30min at room temperature.
3. Remove and discard supernatant. Resuspend pellet in 1ml 8M LiCl. Incubate at 37°C for 15min.
4. Centrifuge at 10,000rpm for 30min at room temperature.
5. Remove and discard supernatant. Resuspend in 1ml 10mM EDTA. Incubate at 37°C for 15min.
6. Wash with dH_2O, acetone and again with dH_2O.
7. Resuspend in 1ml dH_2O.

- Concluding Work:
    - Can be stored at 4°C at this point if desired.
    - The SDS extraction outlined above removes contaminating proteinase K. LiCl and EDTA washes remove materials ionically-bound to the cell wall. Acetone washes removes endotoxins.

Quantification of cell wall prep by crystal violet assay

- Preparatory Work:
    - Prepare some 0.1% crystal violet solution.

1. Add 10µl of 0.1% crystal violet to 90µl of sample and 90µl dH_2O (as a blank).
2. Incubate at room temperature for 1min.
3. Centrifuge at 10,000rpm for 30min.
4. Remove a constant volume of supernatant from both sample and blank.
5. Resuspend with 1ml dH_2O.
6. Measure absorbance at 590nm.

- Concluding Work:
    - The equivalent concentration of *S. pneumoniae* whole bacteria can be found using the standard curve presented in **Figure 1**.
    - Crystal violet is the dye used in Gram staining and binds with peptidoglycan in the cell wall.

RESULTS

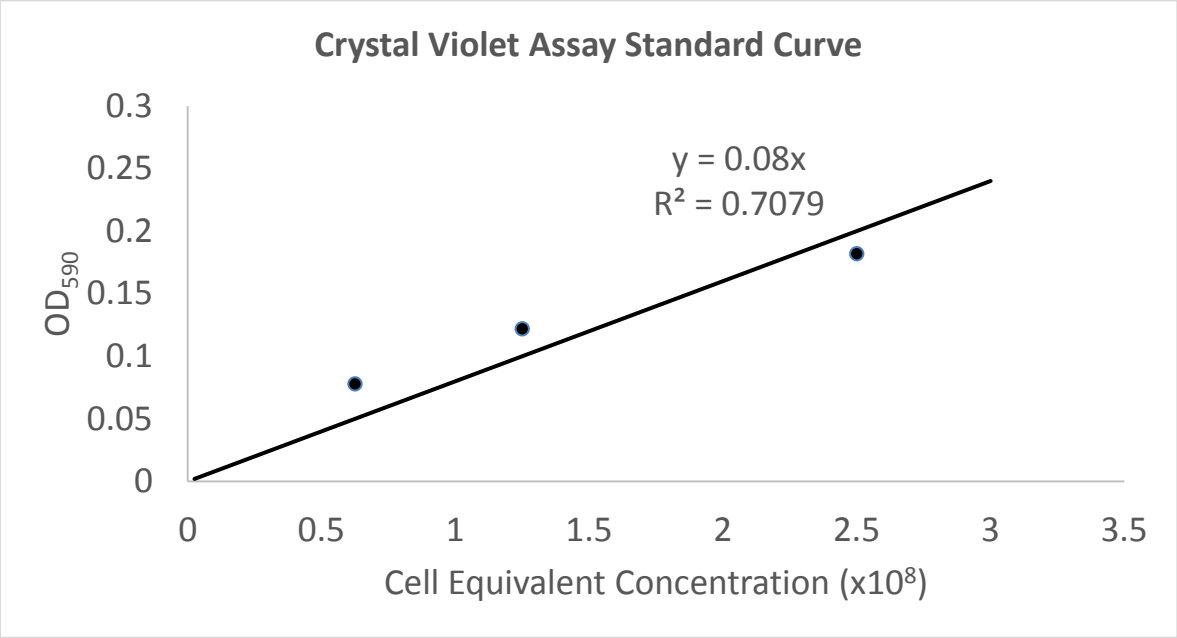

Figure #1: Crystal violet assay whole bacterium standard curve. Standard curve to be used with the crystal violet assay method of quantifying pneumococcal cell wall preparations. Created using a crystal violet assay of whole *S. pneumoniae*.

LINKS AND REFERENCES

1. Tuomanen E, et al. The induction of meningeal inflammation by components of the pneumococcal cell wall. J. Infect. Dis. 151(5): 859-868, 1985. Pubmed: 3989321

2. Bui NK, et al. Isolation and analysis of cell wall components from *Streptococcus pneumoniae*. Anal. Biochem. 421: 657-666, 2012. Pubmed: 22192687