Please cite this article as: Otilia and Christiane, (2016). Antibiotic Disc Assay for *Synechocystis* sp. PCC6803, *Bio-protocol* 6 (24): e2071. DOI: 10.21769/BioProtoc.2071.

bio-protocol www.bio-protocol.org/e2071 Vol 6, Iss 24, Dec 20, 2016
DOI:10.21769/BioProtoc.2071

Antibiotic Disc Assay for Measuring Cell Wall Function in *Synechocystis* sp. PCC6803

Otilia Cheregi* and Christiane Funk*

Department of Chemistry, Umeå University, Umeå, Sweden
*For correspondence: Otilia.cheregi@gmail.com; Christiane.funk@umu.se

[Abstract] This protocol describes how to investigate the integrity of the outer cell wall in the cyanobacterium *Synechocystis* sp. PCC6803 using antibiotics. It is adapted to the agar diffusion test (Bauer *et al.*, 1966), in which filter paper discs impregnated with specified concentrations of antibiotics were placed on agar plates inoculated with bacteria. The antibiotics we tested, interfering with the biosynthesis/function of bacterial cell walls, will diffuse into the agar and produce a zone of cyanobacterial growth inhibition around the disc(s). The size of the inhibition zone reflects the sensitivity of the strain to the action of antibiotics, *e.g.,* a mutation in a protein functioning within the cell wall or its construction would render the mutant strain more sensitive to the respective antibiotic. The method has proven to be useful for phenotyping a mutant of *Synechocystis* sp. PCC6803 lacking all three genes encoding Deg proteases. Deletion of these ATP-independent serine proteases was shown to have impact on the outer cell layers of *Synechocystis* cells (Cheregi *et al.*, 2015).
Keywords: Cyanobacteria, Cell wall, Deg proteases, Antibiotics

[Background] The cyanobacterium *Synechocystis* sp. PCC6803 (hereafter, *Synechocystis* 6803) is a model organism for studying the process of photosynthesis. While its genome was sequenced already in 1996, still more than 50% of its genes encode proteins with hypothetical or unknown function. The three genes *slr1204* (*htrA*), *sll1679* (*hhoA*) and *sll1427* (*hhoB*) encode serine proteases of the Deg (degradation of periplasmic proteins) family; despite detailed analyses (see Cheregi *et al.*, 2016 and references therein) their exact subcellular localization and substrates still are enigmatic. Previous proteomic and metabolomic characterizations of single and triple *deg* deletion mutants performed in our lab have shown altered expression of proteins with functions in or on the outer cell layers of *Synechocystis* 6803 (Miranda *et al.*, 2013; Tam *et al.*, 2015; Cheregi *et al.*, 2015).

The antibiotics carbenicillin, colistin and polymyxin inhibit or disrupt the bacterial cell wall and therefore can be used to test the integrity of this cellular component in mutants: polymyxin acts on the outermost lipopolysaccharide layer surrounding the cyanobacterial S-layer, carbenicillin interferes with the peptidoglycan layer and colistin acts on the plasma membrane of gram-negative bacteria (Table 1). An agar diffusion test has been developed (Bauer *et al.*, 1966) in which filter paper discs impregnated with specified concentrations of antibiotics are placed on agar plates inoculated with bacteria. The antibiotics will diffuse from the disc into the agar and inhibit cyanobacterial growth around it. The size of this inhibition zone then reflects the sensitivity of the strain to the antibiotic. The antibiotic disc assay method was used to characterize a triple *deg* protease mutant, and could be used for the characterization of any cyanobacterial mutant. However, the reader should be aware of the limitations of this assay. Despite the

Please cite this article as: Otilia and Christiane, (2016). Antibiotic Disc Assay for *Synechocystis* sp. PCC6803, *Bio-protocol* 6 (24): e2071. DOI: 10.21769/BioProtoc.2071.

Sll1951 protein being the main component of the outermost cell layer of cyanobacteria, called S-layer, the antibiotic disc assay only had limited effect on a *sll1951* deletion mutant (Trautner and Vermaas, 2013). Though the S-layer is compromised in the *sll1951* deletion mutant, the underlying layers are still intact, preventing Carbenicillin and Polymyxin B, due to their relatively high molecular masses, to penetrate into the cell.

Table 1 describes the molecular weight, the mode of action and the ordering information for the above mentioned antibiotic discs.

Table 1. Antibiotic discs used in this protocol

Disc	MW	Mode of action
Carbenicillin, 100 µg (CAR100)	374.8	Inhibits peptidoglycan biosynthesis
Colistin, 10 µg (CT10)	1155.45	Binds to lipids of cell membrane and disrupts cell wall integrity
Polymyxin B, 300 u (PB300)	1385.61	Binds to lipid A of lypo-polysaccharides and affects membrane permeability

Materials and Reagents

1. Remel™ plastic Petri dishes (85 mm diameter) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: R80085)
2. Sterile polystyrene spreading rods (SARSTEDT, catalog number: 86.1569.005)
3. Carbenicillin (CAR100) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: CT0006B)
4. Colistin (CT10) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: CT0017B)
5. Polymyxin (PB300) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: CT0044B)
6. Dispenser for discs (included in the kit of each of the above mentioned test discs)
7. Cyanobacterial cultures of WT control and mutants to be tested
8. Boric acid, H_3BO_3 (Sigma-Aldrich, catalog number: B6768)
9. Manganese(II) chloride tetrahydrate, $MnCl_2 \cdot 4H_2O$ (Sigma-Aldrich, catalog number: 221279)
10. Zinc sulfate heptahydrate, $ZnSO_4 \cdot 7H_2O$ (Sigma-Aldrich, catalog number: Z1001)
11. Sodium molybdate dehydrate, $Na_2MoO_4 \cdot 2H_2O$ (Sigma-Aldrich, catalog number: 331058)
12. Cupric sulfate pentahydrate, $CuSO_4 \cdot 5H_2O$ (Thermo Fisher Scientific, Fischer Scientific, catalog number: C493-500)
13. Cobalt(II) nitrate hexahydrate, $Co(NO_3)_2 \cdot 6H_2O$ (Sigma-Aldrich, catalog number: 239267)
14. Sodium nitrate, $NaNO_3$ (Scharlab, catalog number: SO05010500)
15. Magnesium sulfate heptahydrate, $MgSO_4 \cdot 7H_2O$ (Sigma-Aldrich, catalog number: 63138)
16. $CaCl_2 \cdot 2H_2O$ (Scharlab, catalog number: CA01981000)
17. Citric acid (Sigma-Aldrich, catalog number: 251275)
18. Na_2-EDTA (Sigma-Aldrich, catalog number: 27285)
19. Ferric ammonium citrate (Sigma-Aldrich, catalog number: F5879)

Please cite this article as: Otilia and Christiane, (2016). Antibiotic Disc Assay for *Synechocystis* sp. PCC6803, *Bio-protocol* 6 (24): e2071. DOI: 10.21769/BioProtoc.2071.

Vol 6, Iss 24, Dec 20, 2016
DOI:10.21769/BioProtoc.2071

20. Sodium carbonate, Na_2CO_3(Sigma-Aldrich, catalog number: 71345)
21. di-potassium hydrogen phosphate, K_2HPO_4 (EMD Millipore, catalog number: 105104)
22. Na-thiosulfate (solid) (Sigma-Aldrich, catalog number: 217263)
23. Difco Bacto-agar (BD, catalog number: 214530)
24. TES (Sigma-Aldrich, catalog number: T6541)
25. 100x BG11 without Fe, phosphate, carbonate (see Recipes)
26. 1,000x ferric ammonium citrate (see Recipes)
27. 1,000x Na_2CO_3 (see Recipes)
28. 1,000x K_2HPO_4 (see Recipes)
29. BG11 solid agar plates (see Recipes)
30. 1 M TES/NaOH buffer, pH 8.2 (see Recipes)

Equipment

1. Cell culture flasks (50-250 ml) with vented caps (TC flask T25) (SARSTEDT, catalog number: 83.3910.002)
2. UV/VIS spectrophotometer (GlobalMarket, PG Instruments, model: T90+) for measuring the absorption of cell culture (OD_{730}).
3. Multisizer™ Coulter Counter for counting cells (Beckman Coulter, model: Z Series Coulter Counter)
4. Laminar hood (Thermo Fisher Scientific, Thermo Scientific™, model: Heraguard™ Eco Clean Bench)
5. Shaking incubator (80-120 rotations/min) with light (I_{VIS} ~60-100 µE m^{-2} s^{-1}) and temperature adjusted to 30 °C (Eppendorf, model: New Brunswick™ Innova® 43)

Procedure

Note: it is recommended that handling of cyanobacterial cultures is done under aseptic conditions using a sterile laminar hood.

1. 100 ml of BG11 media are inoculated with cyanobacterial cells of WT and mutant at an initial OD_{730} ~0.05 and let to grow for 2-3 days until the OD of the cultures measured with the spectrophotometer at 730 nm is between 0.4-0.8.
2. Count cells using the Beckman Coulter cell counter. Alternatively, if a cell counter is not available, cells can be counted using a hemocytometer.
3. Working under sterile conditions, dilute the cyanobacterial cultures with BG11 to 100,000 cells/ml.
4. Spread 1 ml of diluted cultures (containing 100,000 cells) of the WT and mutant strains on Petri dishes containing 40 ml of solid BG11 media respectively. Leave the plates open in the sterile hood until the liquid has evaporated.

Please cite this article as: Otilia and Christiane, (2016). Antibiotic Disc Assay for *Synechocystis* sp. PCC6803, *Bio-protocol* 6 (24): e2071. DOI: 10.21769/BioProtoc.2071.

Vol 6, Iss 24, Dec 20, 2016
DOI:10.21769/BioProtoc.2071

5. One-three antibiotic disks are placed on each plate of the WT and mutant strains. Use at least three biological replicates of each strain to be tested.
6. Petri dishes are placed in an incubator with light and 30 °C and left to incubate (1-2 weeks) until green colonies are visible.
7. Measure the inhibition area around each antibiotic disc to the last mm (Figure 1). If the mutant shows a higher zone of inhibition compared with the WT, this phenotype could be the consequence of the mutation interfering with the biogenesis/function of cell wall.

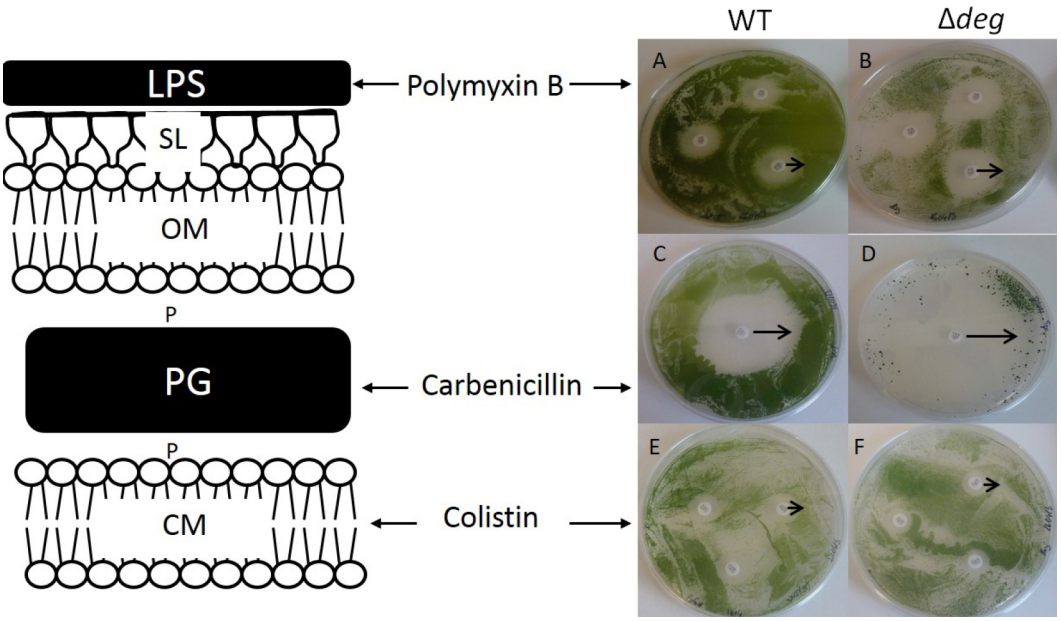

Figure 1. Antibiotic sensitivity test assay. Susceptibility of the WT (A, C, E) and Δ*deg* (B, D, F) to the different antibiotics is shown by their zone of inhibition (ZOI). ZOI induced by Polymixin B is 0.55 (± 0.1) cm and 0.8 (± 0.08) cm in WT (A) and *Δdeg* mutant (B) respectively. ZOI of Carbenicillin was 1.6 (± 0.19) cm for the WT (C) and 2.4 (± 0.25) cm for Δdeg (D). Colistin affected similarly WT (E) and Δdeg (F), ZOI of 0.4 cm. The black arrows indicate the measured zone of inhibitions. Adapted from Cheregi *et al*. (2015).

Data analysis

The average diameters of the areas of inhibition (in cm) and standard deviations were calculated from four biological replicates. Each biological replicate was represented by 3 technical replicates; the technical replicates presented areas of inhibition that were identical. These data are presented in Table 3 and Figure 3 of Cheregi *et al.* (2015).

Please cite this article as: Otilia and Christiane, (2016). Antibiotic Disc Assay for *Synechocystis* sp. PCC6803, *Bio-protocol* 6 (24): e2071. DOI: 10.21769/BioProtoc.2071.

Vol 6, Iss 24, Dec 20, 2016
DOI:10.21769/BioProtoc.2071

Notes

This protocol was adapted from the previously published study of Trautner and Vermaas (2013) and it was performed as in Cheregi *et al.* (2015). The efficiency of the antibiotic is decreasing after expiration date.

Recipes

1. Trace minerals (1 L)
   2.86 g H_3BO_3
   1.81 g $MnCl_2 \cdot 4H_2O$
   0.22 g $ZnSO_4 \cdot 7H_2O$
   0.39 g $Na_2MoO_4 \cdot 2H_2O$
   0.079 g $CuSO_4 \cdot 5H_2O$
   0.049 g $Co(NO_3)_2 \cdot 6H_2O$
2. 100x BG11 without Fe, phosphate, carbonate (1 L)
   149.6 g $NaNO_3$
   7.5 g $MgSO_4 \cdot 7H_2O$
   3.6 g $CaCl_2 \cdot 2H_2O$
   0.60 g citric acid (or 0.89 g Na-citrate, dihydrate)
   1.12 ml 0.25 M Na_2-EDTA, pH 8.0
   100 ml trace minerals
3. Other components
   Ferric ammonium citrate, 6 mg/ml (1,000x): 600 mg per 100 ml dH_2O
   Na_2CO_3 (1,000 x): 2 g Na_2CO_3 per 100 ml dH_2O
   K_2HPO_4 (1,000 x): 3.05 g K_2HPO_4 per 100 ml dH_2O
4. BG11 liquid media (1 L)
   10 ml 100x BG11 without Fe, phosphate, carbonate
   1 ml 1,000x ferric ammonium citrate
   1 ml 1,000x Na_2CO_3
   1 ml 1,000x K_2HPO_4
5. BG11 solid agar plates (1 L)
   For agar plates, add to the above:
   10 ml 1 M TES/NaOH buffer pH 8.2 (long term storage in the fridge)
   3 g Na-thiosulfate (solid)
   15 g Difco Bacto-agar
   Autoclave at 121 °C for 30 min

bio-protocol www.bio-protocol.org/e2071 Vol 6, Iss 24, Dec 20, 2016 DOI:10.21769/BioProtoc.2071

6. 1 M TES/NaOH buffer, pH 8.2
   Dissolve 229.2 gr of TES in distilled H_2O
   The resulting solution should be clear
   Bring the pH to 8.2 using a concentrated solution of NaOH

Acknowledgments

This work was supported by grants from the Swedish Energy Agency (to C. Funk).

References

1. Bauer, A. W., Kirby, W. M. M., Sherris, J. C. and Turck, M. (1966). Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 45(4): 493-496.
2. Cheregi, O., Miranda, H., Gröbner, G. and Funk, C. (2015). Inactivation of the Deg protease family in the cyanobacterium *Synechocystis* sp. PCC 6803 has impact on the outer cell layers. *J Photoch Photobio B* 152: 383-394.
3. Cheregi, O., Wagner, R. and Funk, C. (2016). Insights into the cyanobacterial Deg/HtrA proteases. *Front Plant Sci* 7:624.
4. Miranda, H., Cheregi, O., Netotea, S., Hvidsten, T. R., Moritz, T. and Funk, C. (2013). Co-expression analysis, proteomic and metabolomic study on the impact of a Deg/HtrA protease triple mutant in *Synechocystis* sp. PCC 6803 exposed to temperature and high light stress. *J Proteomics* 78: 294-311.
5. Tam, L. X., Aigner, H., Timmerman, E., Gevaert, K. and Funk, C. (2015). Proteomic approaches to identify substrates of the three Deg/HtrA proteases of the cyanobacterium *Synechocystis* sp. PCC 6803. *Biochem J* 468(3): 373-384.
6. Trautner, C. and Vermaas, W. F. (2013). The *sll1951* gene encodes the surface layer protein of *Synechocystis* sp. strain PCC 6803. *J Bacteriol* 195(23): 5370-5380.

