

Adherent-invasive *Escherichia coli* Biofilm Formation Assays

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[Abstract] Patients with Crohn's disease are abnormally colonized by adherent-invasive *Escherichia coli* (AIEC) bacteria (Chassaing and Darfeuille-Michaud, 2011). These bacteria are able to adhere to and invade intestinal epithelial cells (IEC), to replicate within macrophages, and were recently described to be able to form biofilms (Martinez-Medina *et al.*, 2009; Chassaing and Darfeuille-Michaud, 2013). The reference strain of adherent-invasive *E. coli* is the strain LF82, associated with ileal Crohn's disease (Darfeuille-Michaud *et al.*, 1998).

This protocol described basic steps of a biofilm formation assay on I) non-cell-treated polystyrene microtiter plates and on II) paraformaldehyde-fixed I-407 IEC monolayers.

Materials and Reagents

1. Adherent-invasive *E. coli* reference strain LF82 (Darfeuille-Michaud *et al.*, 1998)
2. Intestine-407 (I-407) cells (ATCC, catalog number: CCL-6)
3. Luria Broth (BD Difco™, catalog number: 244620)
4. Glucose (Sigma-Aldrich, catalog number: G8270)
5. M63 medium (United States Biological, catalog number: M1015)
6. MgSO₄ (Sigma-Aldrich, catalog number: 208094)
7. Phosphate-Buffered Saline (PBS) (Mediatech, Cellgro® , catalog number: 21-040)
8. Crystal Violet (Sigma-Aldrich, catalog number: C6158)
9. 200 proof absolute Ethanol (Sigma-Aldrich)
10. Fetal bovine serum (FBS) (Mediatech, Cellgro®, catalog number: 35-010-CV)
11. Nonessential amino acids (Mediatech, Cellgro®, catalog number: 25-025-CI)
12. L-glutamine (Mediatech, Cellgro®, catalog number: 25-005)
13. Penicillin/Streptomycin/Amphotericin B solution (Mediatech, Cellgro®, catalog number: 30-004-CI)
14. Vitamin mix (Mediatech, Cellgro®, catalog number: 25-020-CI)
15. Formaldehyde solution (Sigma-Aldrich, catalog number: F8775)
16. Phalloidin-tetramethyl rhodamine isocyanate (Sigma-Aldrich, catalog number: P1951)
17. Paraformaldehyde

18. Hoechst 33258 (Sigma-Aldrich, catalog number: B1155)
19. Vectashield (Vector Labs, catalog number: H-1000)
20. Minimum Essential Medium (MEM) (Mediatech, Cellgro[®], catalog number: 10-022-CV) (see Recipes)

Equipment

1. Coverslips (Electron Microscopy Sciences)
2. Sterile tubes
3. 24-well tissue culture plates
4. Non-cell-treated polystyrene 96-well microtiter plates (Falcon[®], catalog number: 62406-117)
5. 24-well polystyrene plate, tissue-culture treated (Falcon[®], catalog number: 62406-159)
6. Microplate shaker (LABREPCO, model: BT1500)
7. Microplate spectrophotometer
8. Confocal microscope
9. 30 °C incubator
10. 37 °C/5% CO₂ incubator

Software

1. Computer program COMSTAT1

Procedure

- A. Biofilm formation assay on non-cell-treated polystyrene microtiter plates
1. Bacterial strains were grown overnight in sterile tubes containing 2 ml of Luria-Bertani broth with 5 g.L⁻¹ glucose at 35.5 °C without agitation (Martinez-Medina *et al.*, 2009; Chassaing and Darfeuille-Michaud, 2013).
 2. The following day, bacterial suspension was diluted 1/100 in M63 minimal medium supplemented with 1 mM of MgSO₄ and 8 g.L⁻¹ glucose.
 3. Aliquots of 130 µl were then placed in wells of non-cell-treated polystyrene 96-well microtiter plates and incubated 24 h at 30 °C without shaking. One well designed as blank received only M63 minimal medium supplemented with 1 mM of MgSO₄ and 8 g.L⁻¹ glucose without bacteria.
 4. After incubation, microplates were agitated for 5 min using a microplate shaker, and bacterial growths were then estimated by OD_{620nm} reading using a microplate spectrophotometer.

5. The liquid was removed by decantation, and the wells were washed once using 200 μ l sterile PBS without agitation.
6. In order to dry them, plates were left open for at least 20 min at room temperature.
7. Adherent bacteria forming biofilm were stained with 130 μ l of 1% crystal violet solubilized in ethanol for 5 min at room temperature without agitation.
8. Wells were then washed five times using 200 μ l sterile PBS without agitation.
9. In order to dry them, plates were left open for at least 1 h at room temperature.
10. 130 μ l of absolute ethanol was added, and plates were incubated 5 min at room temperature without agitation.
11. High-speed agitation was performed using a microplate shaker for 10 min (1000 rpm).
12. Biofilm formation was estimated by OD_{570nm} reading using a microplate spectrophotometer.
13. Specific biofilm formation index could be determined by using the ratio OD_{570nm}/OD_{620nm} that allows standardization of biofilm formation according to bacterial growth in M63 minimal medium. According to previous publication, specific biofilm formation index of wild type AIEC strain LF82 should be between 2 and 4 (Martinez-Medina *et al.*, 2009; Chassaing and Darfeuille-Michaud, 2013).

B. Biofilm formation assay on paraformaldehyde-fixed I-407 IEC monolayers

Intestine-407 (I-407) cells were derived from human intestinal embryonic jejunum and ileum tissues. They were maintained in an atmosphere containing 5% CO₂ at 37 °C in Minimum Essential Medium supplemented with 10% (vol/vol) fetal bovine serum; 1% of nonessential amino acids; 1% of L-glutamine; 1% of Penicillin/Streptomycin/Amphotericin B solution and 1% of vitamin mix.

1. Monolayers were seeded on coverslip in 24-well tissue culture plates with 4 x 10⁵ cells per well and incubated for 20 h at 37 °C with 5% CO₂.
2. Monolayers were then washed once with 1ml PBS, fixed for 15 min in 4% formaldehyde (stock solution diluted in sterile PBS) at room temperature without agitation, and then washed 4 times with PBS.
3. Bacterial strains expressing green fluorescent protein (GFP) (Valdivia *et al.*, 1996) were prepared as previously described above, from step A1 to A2, in M63 minimal medium supplemented with 1 mM of MgSO₄ and 8 g·L⁻¹ glucose.
4. 1 ml of this bacterial suspension was applied on the surface of fixed I-407 cell monolayers, and incubated overnight at 30 °C without shaking.
5. The liquid was removed by decantation, and the wells were washed 3 times using 1 ml of sterile PBS without incubation.
6. I-407/biofilm complex was then fixed for 15 min using 4% formaldehyde.

7. Phalloidin-tetramethyl rhodamine isocyanate was used to visualize actin and Hoechst 33258 was used to visualize nuclei. For this purpose, Phalloidin-tetramethyl rhodamine isocyanate was diluted in sterile PBS at a final concentration of 1µg/ml and Hoechst 33258 was added at a final concentration of 1µg/ml. This solution was added to the wells and incubated for 20 min at room temperature. After incubation, the wells were washed 5 times using 1 ml of sterile PBS without incubation.
8. Coverslips containing the fixed and stained I-407/biofilm complex were then removed from the 24-well plate and mounted on slide using Vectashield.
9. The slides were examined with a confocal microscope. An example of biofilm formation on paraformaldehyde-fixed I-407 IEC monolayers is presented Figure 1.
10. Images from biofilm formation assay at the surface of intestinal epithelial cells I-407 monolayers could be analyzed for thickness and/or roughness with the computer program COMSTAT1 (Haydorn *et al.*, 2000).

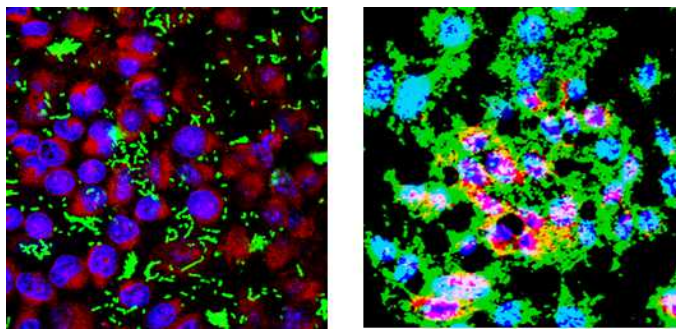


Figure 1. Confocal analysis of biofilm formation of a poor biofilm producer (Non pathogenic *Escherichia coli* K12 strain C600 (A) and a strong biofilm producer (Adherent-Invasive *Escherichia coli* strain LF82 (B) at the surface of a PFA-fixed monolayer of I-407 intestinal epithelial cells. Bacteria express GFP (green), actin is stained in red using phalloidin-TRITC, and DNA is stained in blue using Hoechst. Bars, 50 µm.

Notes

1. Due to high variation observed with both of these biofilm formation assays, they need to be performed at least in triplicate, including controls.

Recipes

1. Minimum Essential Medium supplemented with 10% (vol/vol) fetal bovine serum

- 1% of nonessential amino acids
- 1% of L-glutamine
- 1% of Penicillin/Streptomycin/Amphotericin B solution
- 1% of vitamin mix

Acknowledgments

This study was supported by the Ministère de la Recherche et de la Technologie, the Institut National de la Santé et de la Recherche Médicale and the Université d'Auvergne (UMR Inserm 1071), the Institut National de la Recherche Agronomique (USC INRA 2018) and by grants from the Association F. Aupetit (AFA). We thank the CICS platform for confocal microscopy. This protocol is adapted from previously published papers (Danese *et al.*, 2000; Naves *et al.*, 2008; Martinez-Medina *et al.*, 2009; Chassaing and Darfeuille-Michaud, 2013). This work is dedicated to Arlette Darfeuille-Michaud.

References

1. Chassaing, B. and Darfeuille-Michaud, A. (2011). [The commensal microbiota and enteropathogens in the pathogenesis of inflammatory bowel diseases.](#) *Gastroenterology* 140(6): 1720-28.
2. Chassaing, B. and Darfeuille-Michaud, A. (2013). [The sigmaE pathway is involved in biofilm formation by Crohn's disease-associated adherent-invasive *Escherichia coli*.](#) *J Bacteriol* 195(1): 76-84.
3. Danese, P. N., Pratt, L. A., Dove, S. L. and Kolter, R. (2000). [The outer membrane protein, antigen 43, mediates cell-to-cell interactions within *Escherichia coli* biofilms.](#) *Mol Microbiol* 37(2): 424-432.
4. Darfeuille-Michaud, A., Neut, C., Barnich, N., Lederman, E., Di Martino, P., Desreumaux, P., Gambiez, L., Joly, B., Cortot, A. and Colombel, J. F. (1998). [Presence of adherent *Escherichia coli* strains in ileal mucosa of patients with Crohn's disease.](#) *Gastroenterology* 115(6): 1405-1413.
5. Heydorn, A., Nielsen, A. T., Hentzer, M., Sternberg, C., Givskov, M., Ersboll, B. K. and Molin, S. (2000). [Quantification of biofilm structures by the novel computer program COMSTAT.](#) *Microbiology* 146 (Pt 10): 2395-2407.
6. Martinez-Medina, M., Naves, P., Blanco, J., Aldeguer, X., Blanco, J. E., Blanco, M., Ponte, C., Soriano, F., Darfeuille-Michaud, A. and Garcia-Gil, L. J. (2009). [Biofilm formation as a novel phenotypic feature of adherent-invasive *Escherichia coli* \(AIEC\).](#) *BMC Microbiol* 9: 202.

7. Naves, P., del Prado, G., Huelves, L., Gracia, M., Ruiz, V., Blanco, J., Dahbi, G., Blanco, M., Ponte Mdel, C. and Soriano, F. (2008). [Correlation between virulence factors and in vitro biofilm formation by *Escherichia coli* strains](#). *Microb Pathog* 45(2): 86-91.
8. Valdivia, R. H., Hromockyj, A. E., Monack, D., Ramakrishnan, L. and Falkow, S. (1996). [Applications for green fluorescent protein \(GFP\) in the study of host-pathogen interactions](#). *Gene* 173(1 Spec No): 47-52.