

Structural Analysis of *Bordetella pertussis* Biofilms by Confocal Laser Scanning Microscopy

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[Abstract] Biofilms are sessile communities of microbial cells embedded in a self-produced or host-derived exopolymeric matrix. Biofilms can both be beneficial or detrimental depending on the surface. Compared to their planktonic counterparts, biofilm cells display enhanced resistance to killing by environmental threats, chemicals, antimicrobials and host immune defenses. When in biofilms, the microbial cells interact with each other and with the surface to develop architecturally complex multi-dimensional structures. Numerous imaging techniques and tools are currently available for architectural analyses of biofilm communities. This allows examination of biofilm development through acquisition of three-dimensional images that can render structural features of the sessile community. A frequently utilized tool is Confocal Laser Scanning Microscopy. We present a detailed protocol to grow, observe and analyze biofilms of the respiratory human pathogen, *Bordetella pertussis* in space and time.

Keywords: Biofilm, Confocal microscopy, *Bordetella pertussis*, Whooping cough, Biofilm-structure

[Background] *Bordetella pertussis* is an obligate human pathogen of the upper respiratory tract that causes whooping cough or pertussis (Mooi, 2010; Dorji *et al.*, 2018). Biofilms of *B. pertussis* form on a variety of artificial surfaces and under static, shaking, and fluid flow conditions (Mishra *et al.*, 2005; Sloan *et al.*, 2007; Serra *et al.*, 2011). Microscopic evaluation of these biofilms shows that this bacterium produces irregularly shaped microcolonies separated by fluid channels, embedded in an exopolymeric matrix composed by extracellular DNA (eDNA), proteins and polysaccharides (Parise *et al.*, 2007; Sloan *et al.*, 2007; Serra *et al.*, 2008; Conover *et al.*, 2011; Nicholson *et al.*, 2012; Ganguly *et al.*, 2014; Cattelan *et al.*, 2017). In addition to forming biofilms in the laboratory setting, *B. pertussis* forms multi-dimensional organ-adherent biofilms on the nose and trachea during experimental infections of mice. Development of these mammalian biofilms is characterized by an extracellular polymeric matrix composed of eDNA, the Filamentous hemagglutinin protein and the Bps polysaccharide (Conover *et al.*, 2010 and 2011; Serra *et al.*, 2011; Dorji *et al.*, 2018). Based on these results, biofilm formation has been proposed by us and others as a possible strategy adopted by *B. pertussis* to infect, persist and continually circulate in the community (Cattelan *et al.*, 2016). Consistent with this hypothesis, we found that currently circulating strains from Argentina and USA produce significantly higher levels of biofilms when compared to a laboratory reference strain and colonize the mouse nose and trachea at higher

numbers than the prototype laboratory strain (Cattelan *et al.*, 2017). These results also provide evidence that hyperbiofilm growth is a strategy employed by circulating organisms to infect and survive inside their host.

In order to study the mechanisms involved in biofilm development, microscopic evaluation is a key technique that allows differentiation of the steps of the process, from adhesion to maturation and dispersion. In particular, confocal laser scanning microscopy (CLSM) is frequently used because it allows visualization of architectural complexities of intact and hydrated biofilms. In this protocol, we describe how to grow and process samples of *B. pertussis* biofilms, as demonstrated in our recent publication (Cattelan *et al.*, 2017).

Materials and Reagents

1. 0.2 µm filter
2. Bacteriological Petri plates (Fisher Scientific, catalog number: FB0875712)
3. Sterile test tubes (17 x 100 mm, RPI, Research Products International, catalog number: 168599)
4. Serological pipettes:
 - 5 ml pipette (SARSTEDT, catalog number: 86.1253.001)
 - 25 ml pipette (SARSTEDT, catalog number: 86.1685.001)
5. Nalgene™ 2 ml cryogenic tubes (Thermo Fisher Scientific, Thermo Scientific™, catalog number 5000-0020)
6. Coverglasses, 22 x 22 mm (Fisher Scientific, Fisherbrand™, catalog number: 12-542B)
7. Nunc™ 6-wells plates (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 150239)
8. Aluminum foil
9. Microscope slides (Fisher Scientific, Fisherbrand™, catalog number: 12-552-3)
10. Sterile disposable plastic material:
 - Posi-Click tubes (Denville Scientific, catalog number: C2170)
 - P200 and P1000 tips (USA Scientific, catalog numbers: 1111-0706 and 1112-1720)
11. *B. pertussis* strain harboring a pGB5P1-GFP plasmid
12. Defibrinated sheep's blood (Hemostat Laboratories, catalog number: DSB100)
13. 50% glycerol solution (Fisher Scientific, catalog number: G33-1)
14. Kanamycin (Sigma-Aldrich, catalog number: K1377)
15. Neutralized buffered formalin (Fisher Scientific, catalog number: SF100-4)
16. Stainer-Scholte medium (Stainer *et al.*, 1970; Nicholson *et al.*, 2012)
17. Sterile PBS (Thermo Fisher Scientific, Gibco™, catalog number: 14190144)
18. ProLong™ Gold Antifade Mountant (Thermo Fisher Scientific, Invitrogen™, catalog number: P36934)
19. Difco™ Bordet-Gengou agar plates (BD, BD Biosciences, catalog number: 248200)
20. L-Proline (Sigma-Aldrich, catalog number: 131547)
21. KH₂PO₄ (Sigma-Aldrich, catalog number: V000225)

22. KCl (Sigma-Aldrich, catalog number: P3911)
23. MgCl₂·6H₂O (Sigma-Aldrich, catalog number: M2393)
24. CaCl₂ (Sigma-Aldrich, catalog number: C1016)
25. Tris base (Sigma-Aldrich, catalog number: T1378)
26. NaCl (Sigma-Aldrich, catalog number: S7653)
27. FeSO₄·7H₂O (Sigma-Aldrich, catalog number: F8633)
28. L-cystine (Sigma-Aldrich, catalog number: C8755)
29. L-ascorbic acid (Sigma-Aldrich, catalog number: A5960)
30. Nicotinic acid (Sigma-Aldrich, catalog number: PHR1276)
31. Reduced L-glutathione (Sigma-Aldrich, catalog number: G4251)
32. SS medium (see Recipes)
33. SS supplement (filter-sterilized) (see Recipes)
34. Bordet-Gengou (BG) medium (see Recipes)

Equipment

1. Kimax® Erlenmeyer flasks (125 ml) (DWK Life Sciences, KIMBLE®, catalog number: 26500)
2. Sterile forceps
3. PIPETMAN® Classic (Gilson, models: P20, P200, P1000, catalog numbers: F123600, F123601, F123602)
4. Humid chamber (an appropriately sized Tupperware container with either a weigh boat containing water or paper towels soaked with water)
5. Chemical fume hood (Hamilton Laboratory Solutions, model: PL-822)
6. Eclipse Ti-E inverted Confocal Microscope (Nikon, model: D-Eclipse C1si)
7. Vortex (Fisher Scientific, catalog number: 02-215-365)
8. pH meter (Denver Instrument, model: UB-10)
9. Incubator (Thermo Fisher Scientific, Thermo Scientific™, model: Forma™ Steri-Cycle™)
10. Benchtop centrifuge (Eppendorf, model: 5418)
11. Refrigerator (4 °C) (Whirlpool, model: WRR56X18FW02)
12. -20 °C freezer (Kenmore, model: 253-26722103)
13. SPECTRONIC™ Spectrophotometer (Thermo Fisher Scientific, model: GENESYS 20)
14. Weigh Balance (Ohaus, model: Scout Pro SP202 and VWR, model: VWR-164AC)
15. Roller Drum (Eppendorf, New Brunswick™, model: TC-2)

Software

1. ImageJ (Schneider *et al.*, 2012)
2. COMSTAT2 plugin (Heydorn *et al.*, 2000)

Procedure

Notes:

- The entire protocol is depicted in Figure 1.
- Samples should be protected from light during the experiment. In all cases, medium is supplemented with appropriate antibiotic to maintain the plasmid coding for GFP.

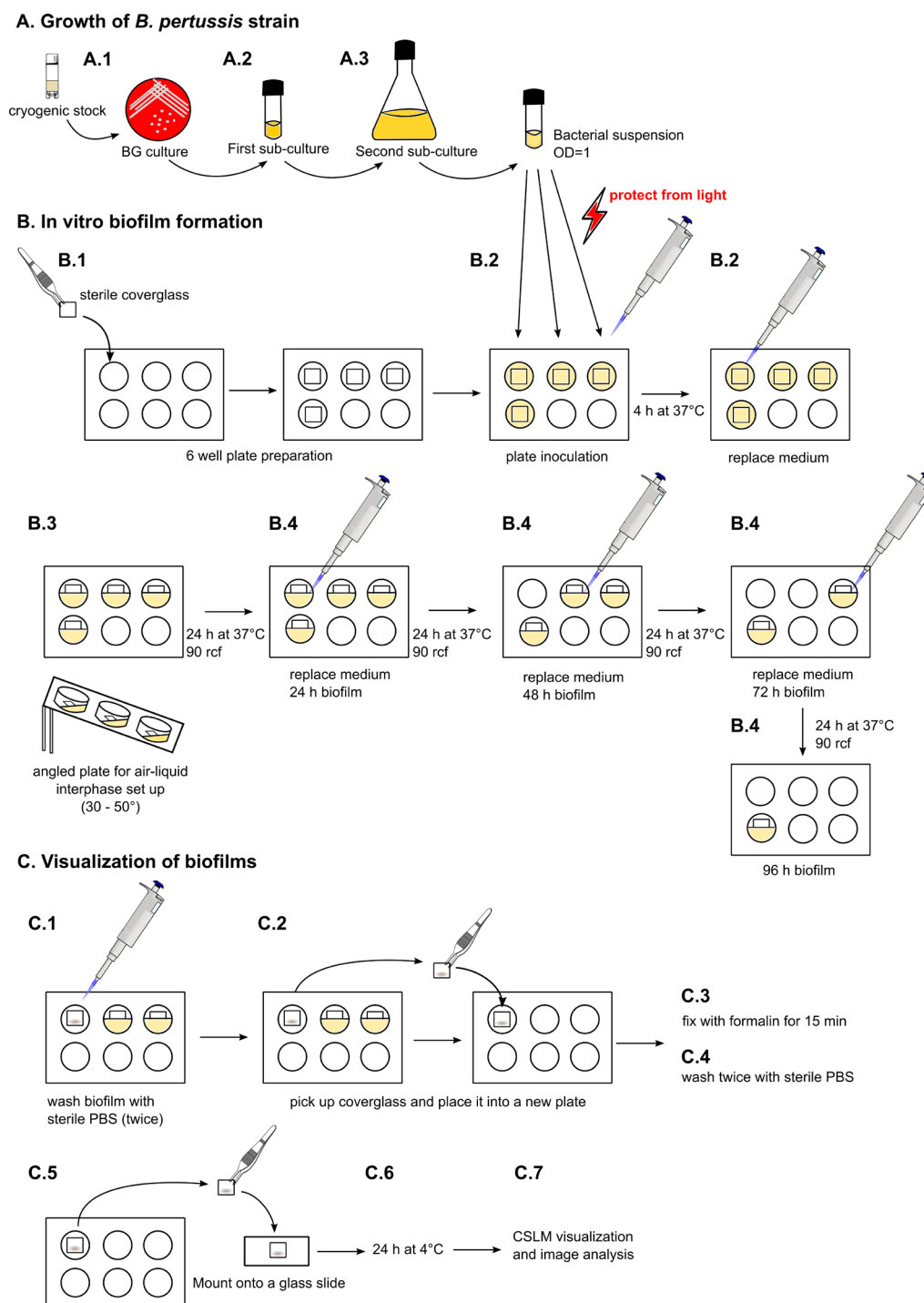


Figure 1. Schematic step-by-step protocol for growing *B. pertussis* biofilms for CSLM

evaluation

A. Growth of *B. pertussis* strains

1. From frozen stock stored at -78 °C, streak *B. pertussis* strains harboring a GFP-coding plasmid, pGB5P1-GFP was used in this protocol (Weingart *et al.*, 1999), on Bordet-Gengou agar plates supplemented with 10% defibrinated sheep's blood and 25 µg/ml of kanamycin. Incubate the plates at 37 °C for 4-5 days.
2. Pick individual colonies (~8-10) and inoculate 2 ml of Stainer-Scholte (SS) liquid medium supplemented with 25 µg/ml of kanamycin. Incubate at 37°C in a roller drum spinning at top speed for 24 h.
3. Inoculate 30 ml of SS medium supplemented with 25 µg/ml of kanamycin (in a 100 ml Erlenmeyer flask), with the cultures from Step A2 to achieve an OD₆₅₀ of 0.2. Incubate for 12 h at 37 °C with shaking at 160 rcf.

B. *In vitro* biofilm formation

1. Insert a sterile coverglass (flame sterilized) at the bottom of sterile 6-well plates.
2. Prepare inoculum by diluting *B. pertussis* culture (Procedure A, Step 3) to an OD₆₅₀ of 1.0 in SS medium. Pipet 1.5 ml of inoculum into individual wells of the 6-well plates. Make sure the bacterial suspension is covering the entire surface of the coverslip. In case other substrates are used (*i.e.*, polycarbonate or aluminum coupons), volume should be adjusted according to the thickness of the material.
3. Cover the plate and incubate statically in a humidified chamber for 4 h at 37 °C.
Note: This step allows the adhesion of bacteria to the surface.
4. Remove inocula (to allow removal of unattached planktonic bacteria) carefully without disturbing attached cells and add approximately 800 µl of fresh SS medium, dispensing along the wall of the well to prevent biofilm disruption. Place the plate at an angle of 30°-50° relative to horizontal, forming an air-liquid interface in the coverglass where biofilms will develop. Cover the plate with aluminum foil and incubate in a humidified chamber at 37 °C on a platform shaker at 90 rcf.
5. If the biofilm formation is going to be examined for longer than 24 h, change medium every 24 h as described above. Samples can be processed at 24, 48, and 72 up to 96 h.

C. Visualization of biofilms

1. After each time point, aspirate medium from each well and carefully wash the biofilms twice with sterile PBS.
2. Pick the coverglass up with forceps and place it into a new 6-well plate. A needle can be used to aid in lifting the coverglass followed by use of forceps to pick the coverglass without touching the area where the biofilm developed.
3. Carefully add 50 µl of neutral buffered formalin, or enough to cover the glass surface. Special attention must be taken in order to not disturb the biofilm and incubate for 15 min at room

- temperature.
4. Remove fixative and wash twice with PBS.
5. Pick the coverglass up with forceps and mount onto a glass slide with ProLong™ Gold mounting media. Alternatively, if bacteria are not labeled with a fluorescent protein, cells can be stained at this point with a fluorescent dye and then mounted.
6. Allow the mounting media to harden by incubating at 4 °C for 24 h.
7. Slides can be stored (weeks to months) in the dark at room temperature for later visualization. Examine the samples with a confocal microscope, taking images in a z-stack configuration, making sure to cover the entire biofilm structure from bottom to top.
8. Analyze the images by using microscopy image visualization software (Figure 2).

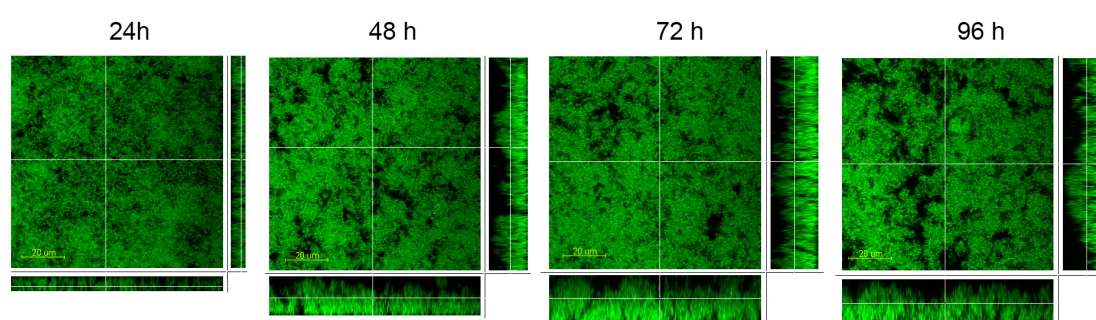


Figure 2. Micrographs of a *B. pertussis* biofilm. Biofilms were grown from 24 up to 96 h and images were acquired in an inverted confocal microscope in a z-stack setting. Images are presented in xy, xz and yz planes.

Data analysis

Quantitative data from images can be obtained with COMSTAT2 plug-in (Heydorn *et al.*, 2000) run in ImageJ (Schneider *et al.*, 2012). Structural features that can be analyzed are maximum and average thicknesses, substrate coverage, biomass and roughness coefficient (see structural data in Table 1). Detailed instructions for COMSTAT2 use are described in Heydorn *et al.* (2000). Appropriate statistical analysis should be carried out on the obtained data.

Table 1. Biofilm structural features of the images presented in Figure 2, obtained with COMSTAT2 software

	24 h	48 h	72 h	96 h
Biomass ($\mu\text{m}^3/\mu\text{m}^2$)	2.05	3.70	5.73	4.41
Average thickness (μm)	5.62	12.20	20.78	15.66
Maximum thickness (μm)	7.00	18.00	28.00	19.00
Roughness coefficient	1.10	1.06	1.04	1.06

Notes

1. Due to variations observed with this assay, there is need to be performed with duplicates of each sample and at least three independent replicates. For each sample, at least three representative images should be acquired.
2. A critical consideration is not to disrupt the biofilm attached to the glass surface. Depending on the microorganism, cells may attach either strongly or loosely to surfaces. Thus it is important to be extremely careful while dispensing liquid to avoid strong shear forces.
3. BG-agar medium and SS medium are sterilized by autoclaving at 121 °C for 15 min.
4. To prepare a cryogenic stock, cultures grown to exponential phase (set up as explained in Step A2) should be used. Use sterile 2 ml cryogenic tubes and mix 500 µl of bacterial culture with 500 µl of sterile 50% glycerol solution. Store at -78 °C.

Recipes

Note: Unless otherwise indicated, all stock solutions are prepared using Milli Q water.

1. SS medium
Monosodium glutamate: 10.70 g/L
L-Proline: 0.24 g/L
KH₂PO₄: 0.5 g/L
KCl: 0.20 g/L
MgCl₂·6H₂O: 0.20 g/L
CaCl₂: 0.2 g/L
Tris base: 1.52 g/L
NaCl: 2.50 g/L
Adjust to pH 7.3-7.4 with HCl
2. SS supplement (0.2 µm filter-sterilized)
Dissolve in HCl concentrated, and then filter sterilize
FeSO₄·7H₂O: 10 mg/L
L-cystine: 40 mg/L
L-ascorbic acid: 20 mg/L
Nicotinic acid: 4 mg/L
Reduced L-glutathione: 100 mg/L
3. Bordet-Gengou (BG) medium
Difco™ Bordet-Gengou agar (30 g/L)
20 ml of 50% glycerol
Autoclave at 121 °C for 15 min
Aseptically add 15% sterile, defibrinated blood to the medium at 45-50 °C

Mix well and plate

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The authors declare no conflict of interest.

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