Video Article

Growth of *Mycobacterium tuberculosis* Biofilms

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Abstract

Mycobacterium tuberculosis, the etiologic agent of human tuberculosis, has an extraordinary ability to survive against environmental stresses including antibiotics. Although stress tolerance of *M. tuberculosis* is one of the likely contributors to the 6-month long chemotherapy of tuberculosis [1], the molecular mechanisms underlying this characteristic phenotype of the pathogen remain unclear. Many microbial species have evolved to survive in stressful environments by self-assembling in highly organized, surface attached, and matrix encapsulated structures called biofilms [2-4]. Growth in communities appears to be a preferred survival strategy of microbes, and is achieved through genetic components that regulate surface attachment, intercellular communications, and synthesis of extracellular polymeric substances (EPS) [5,6]. The tolerance to environmental stress is likely facilitated by EPS, and perhaps by the physiological adaptation of individual bacilli to heterogeneous microenvironments within the complex architecture of biofilms [7].

In a series of recent papers we established that *M. tuberculosis* and *Mycobacterium smegmatis* have a strong propensity to grow in organized multicellular structures, called biofilms, which can tolerate more than 50 times the minimal inhibitory concentrations of the anti-tuberculosis drugs isoniazid and rifampicin [8-10]. *M. tuberculosis,* however, intriguingly requires specific conditions to form mature biofilms, in particular 9:1 ratio of headspace: media as well as limited exchange of air with the atmosphere [9]. Requirements of specialized environmental conditions could possibly be linked to the fact that *M. tuberculosis* is an obligate human pathogen and thus has adapted to tissue environments. In this publication we demonstrate methods for culturing *M. tuberculosis* biofilms in a bottle and a 12-well plate format, which is convenient for bacteriological as well as genetic studies. We have described the protocol for an attenuated strain of *M. tuberculosis*, *mc^{2}7000*, with deletion in the two loci, *panCD* and *RD1,* that are critical for *in vivo* growth of the pathogen [9]. This strain can be safely used in a BSL-2 containment for understanding the basic biology of the tuberculosis pathogen thus avoiding the requirement of an expensive BSL-3 facility. The method can be extended, with appropriate modification in media, to grow biofilm of other culturable mycobacterial species.

Overall, a uniform protocol of culturing mycobacterial biofilms will help the investigators interested in studying the basic resilient characteristics of mycobacteria. In addition, a clear and concise method of growing mycobacterial biofilms will also help the clinical and pharmaceutical investigators to test the efficacy of a potential drug.

Video Link

The video component of this article can be found at http://www.jove.com/video/3820/

Protocol

1. Growing biofilms of *M. tuberculosis* in a 250mL screw capped bottle

1. Media preparation: Dissolve 0.5g of KH_2PO_4, 0.5g of $MgSO_4$, 4g of L-Asparagine, 2g of Citric acid, 0.05g of Ferric Ammonium Citrate, 60mL of glycerol in 900mL of water. Adjust the pH to 7.0 with NaOH. Autoclave, cool and just prior to starting the experiment, add sterile $ZnSO_4$ to a final concentration of 0.1% w/v. Since *mc^{2}7000* is a pantothenate auxotroph this strain also requires pantothenic acid at 10µg/mL of final concentration.

Note: This is a standard composition of Sauton's media used for M. tuberculosis. However, if required other specialized media can also be used for other mycobacterial species.

2. Inoculums preparation: Grow *M. tuberculosis* in 7H9OADC with 0.05% Tween-80 for one week, or OD_{600} of 0.7 to 1.0. The culture can be directly used as inoculum at a 1:100 dilution.
3. Dispense 25mL of Sauton's media to a 250mL screw capped polystyrene bottle (Corning). Add 250µl of the inoculum to the medium, cap the bottle very tightly and place it undisturbed in a humidified 37°C incubator for 3 weeks. Observe the bottle once every day to ensure that there is no contamination.
4. At the end of third week, loosen the cap of the bottle to allow further growth of *M. tuberculosis* at the interface. If the cap is not loosened at this stage then the insufficient oxygen concentration in the container will retard further growth of bacteria.

2. Growth of *M. tuberculosis* biofilms in 12-well plates

1. Prepare the media and inoculum of *mc²7000* as described in sections A1 and A2.
2. Mix 60mL of media with 600µl of inoculum. Dispense 4.5mL of the mixture into each well of the plate. Cover the plate with lid. Wrap the plate several times with parafilm. Incubate the plate undisturbed in humidified incubator at 37°C for 5 weeks.

3. Determining the frequency of drug tolerant persisters in *M. tuberculosis* biofilms

1. Grow *M. tuberculosis* biofilms in a 12-well format as described in section B. This will take a total of about 5-weeks.
2. Once the biofilms are matured (after 5 weeks of incubation) inject your choice of antibiotic at desired concentration into the media beneath the biofilms using a microtip in a pipette.

Note: The volume of the media beneath the pellicles reduces to about 3.0mL. So investigators should accordingly calculate the amount of drug.

3. Swirl the plate gently so that the antibiotic is thoroughly diffused in the medium. For statistically significant results, inject the antibiotic in four wells. In parallel, inject the same volume of solvent in which the antibiotic was dissolved in other four wells, and leave the last four wells of the plate untouched. Cover the plate with lid and put fresh layers of parafilm around the plate. Place it back in the incubator for desired time period.
4. At the end of the incubation, open the plate and add Tween-80 to the final concentration of 0.1% (volume/volume) in each of the wells. Swirl the whole plate gently for uniform dispersal of the detergent. Incubate the plate at room temperature for 15 minutes. Mix the content of each well with pipette several times so that entire content can be uniformly transferred to a 15mL conical tube.
5. Spin down the content of the tube at 4000rpm for 10 minutes at room temperature. Resuspend the pellet in 5mL of fresh wash buffer (PBS with 10% glycerol and 0.05% Tween-80). Repeat the washing three times. Resuspend the pellet in 5mL of wash buffer. Keep it on the rocker for overnight at 4°C.

Note: Although low temperature was originally developed for M. smegmatis (to minimize its growth during dispersion) and used for M. tuberculosis as well, the slow growing species can most likely be rocked at room temperature without any impact on result. Rocking at room temperature could be necessary if working in BSL-3 facility.

6. Prepare a sterile syringe fitted with microtip (2-200µl) by cutting its broad end to the appropriate size, fitting it to the syringe and wrapping it with parafilm. Pass the whole content of the tube through the tip-fitted syringe and collect in a fresh 15mL tubes. Repeat this step 5 to 6 times till you observe a fairly homogenous suspension.
7. Prepare serial dilutions of the suspension and plate the dilutions on 7H11OADC plate to determine the number of viable colonies in each well. Incubate the plates for three weeks in 37°C incubator. Determine the frequency of persisters in the biofilm population by calculating the ratio of number of colonies obtained in antibiotic treated to those obtained on solvent treated plates.

4. Representative Results

When cultured in a bottle, growth of *M. tuberculosis* can be seen at the base of the bottle by the end of the first week. By the end of the second week, patchy growth of bacteria on the air-media interface can be seen, although growth at the air-media interface is consistently visible at the end of the third week (Fig. 1A). At this time the attachment of the bacteria to the wall of the container is also observed. From this point onward the growth of the culture primarily occurs on the air-media interface. The liquid beneath the surface growth is clear. Typically, the structure matures by the end of the fifth week (Fig. 1B). If incubation is prolonged, the structures will begin to sink to the bottom of the container. Intriguingly, tightening of the cap until the end of third week is an important step in the process, and for unknown reasons a loose-capped bottle significantly retards initiation of growth on the interface [9].

In the 12-well format, a robust biofilm at the air-media interface is seen in each of the wells at the end of five weeks (Fig 2A). If the plates are not wrapped completely then differential biofilm growth is observed. In the worst case, significant media evaporation can stall the growth of the bacteria (Fig 2B). Thus, wrapping of the plate is necessary both to prevent the evaporation as well as to provide environment for biofilm formation (see previous paragraph).

The number of viable bacilli in biofilms determined with this technique is quite reproducible. Response of *M. tuberculosis* biofilms varies with the nature of the antibiotics. For a bactericidal antibiotic such as rifampicin, loss of viability follows a biphasic trend [9]. A rapid decline in the viability during the first three to four days followed by a persistent phase in which a small percentage of the population remain completely recalcitrant to antibiotics irrespective of the concentration of the antibiotic or the time of exposure. Figure 3 shows the number of viable bacilli in mature biofilms after a 7-day exposure to 50µg/mL (50 times higher than the MIC) of rifampicin.

Figure 1A. Early appearance of *M. tuberculosis* bacilli on the air-media interface of bacteria after 3 weeks of incubation.

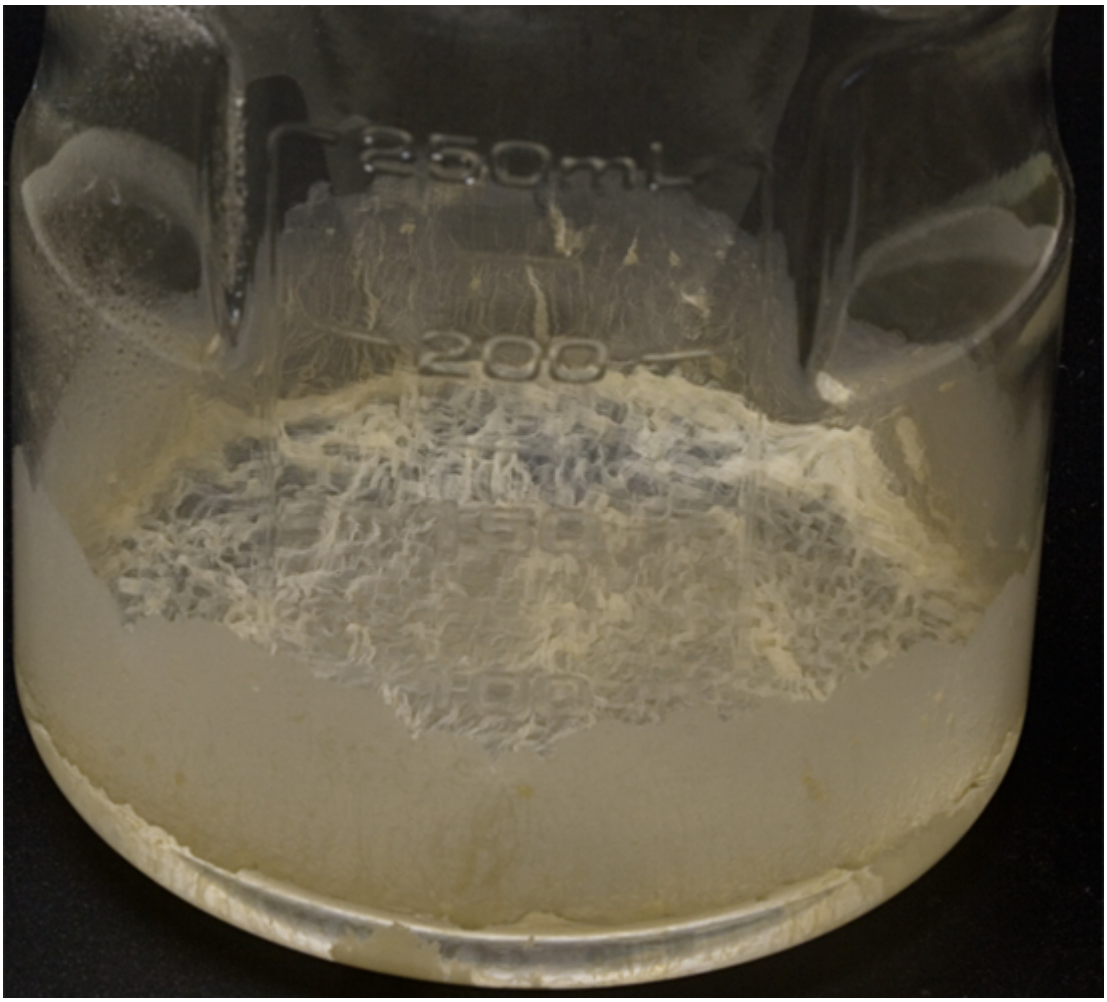

Figure 1B. Matured biofilms of *M. tuberculosis* on the air-media interface after five weeks of incubation.

Figure 2A. 5-week old biofilms of *M. tuberculosis* grown in 12-well format.

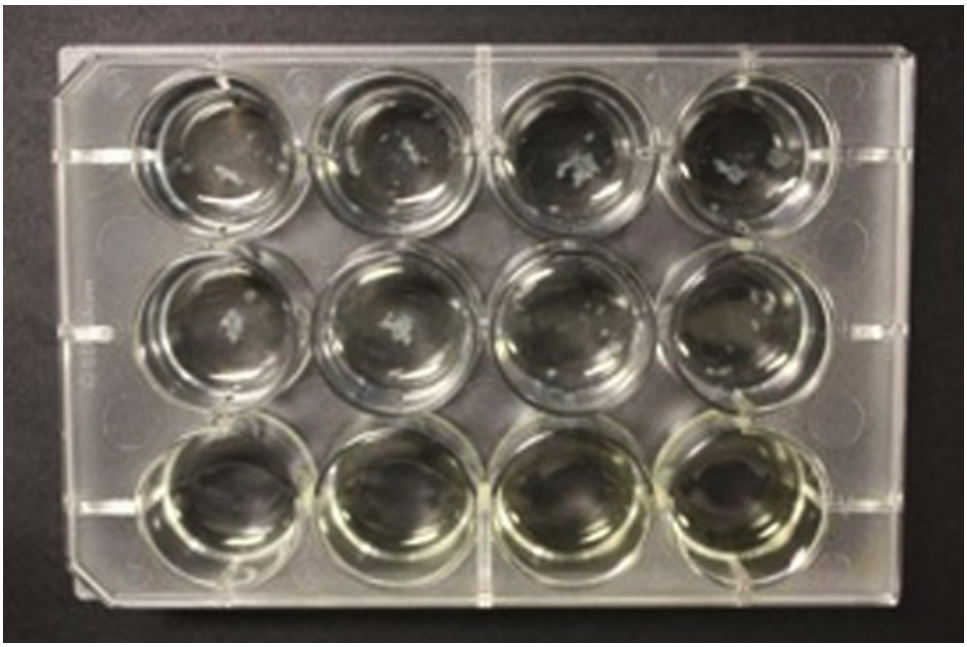

Figure 2B. A failed attempt to grow M. tuberculosis biofilms in 12-well plate without parafilm.

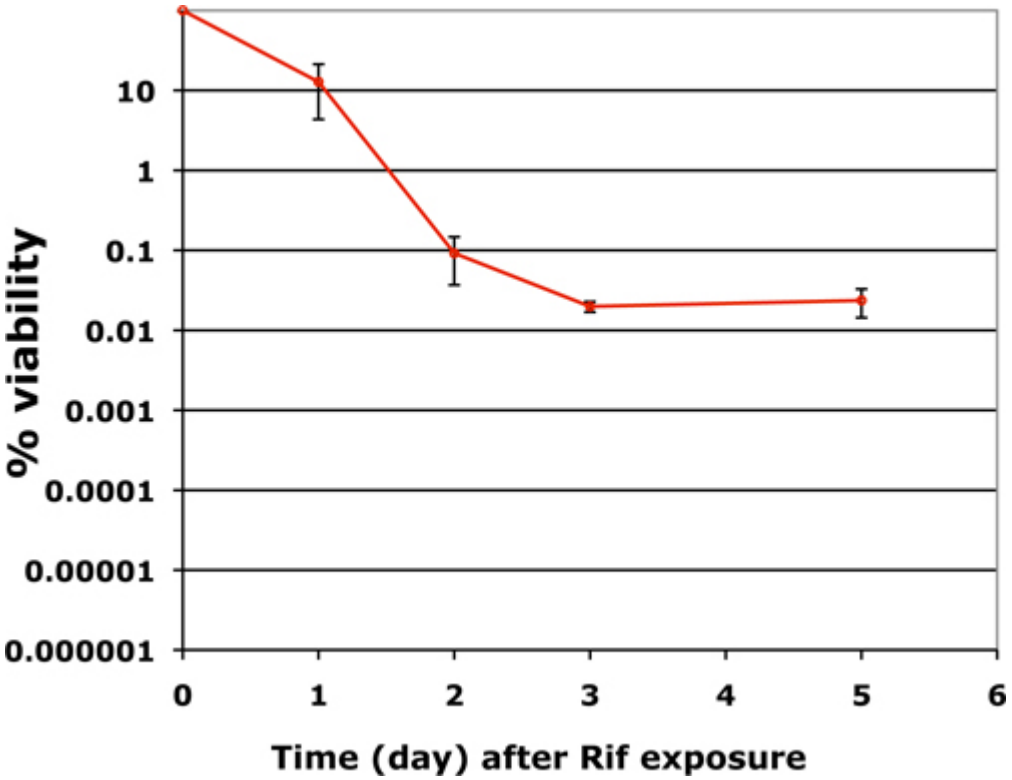

Figure 3. A representative plot showing the frequency of drug tolerant persisters in *M. tuberculosis* biofilms grown in 12-well format and exposed to 50µg/mL of Rifampicin for seven days.

Discussion

Tuberculosis (TB), caused by the infection of *Mycobacterium tuberculosis*, remains a major threat to the global public health. Nearly one third of the world's population is estimated to be asymptomatically infected by the pathogen, about 9 million new cases show up in clinic every year with symptoms of active TB and about 1.7 million die of the infection every year [11]. The huge burden of the disease is primarily contributed by lack of a vaccine and a highly complicated chemotherapy that involves a multidrug regimen administered over six to nine months. The prolonged chemotherapy is largely attributed to phenotypic tolerance of a small subpopulation of the pathogen that required extended exposure of antibiotics [12]. While targeting these persisters is critical for short and effective treatment of tuberculosis, the mechanisms underlying the development of these persisters remain unclear.

Most microbial species in their natural habitat spontaneously grow in self-assembled, surface attached multicellular communities called biofilms, which are highly tolerant to antibiotics[3]. Recently, it has been shown that several mycobacterial species, have strong propensity to grow *in vitro* as biofilms, which provide a microenvironment that promote development of drug tolerant persisters [9,13-15]. While fast growing environmental species such as *M. smegmatis* can readily form biofilms in detergent-free media, the slow growing pathogenic species *M. tuberculosis* require specific conditions to form the multicellular structures [9]. Since culturing *M. tuberculosis* in biofilms could provide significant insight into the mechanisms of drug tolerance and persistence, dissemination of a detailed protocol for culturing the pathogen in biofilms through an open source will be valuable for tuberculosis research, particularly in a resource-limited country.

Here we describe the method of growing *M. tuberculosis* in biofilms in detail. We also provide a protocol to break the biofilms and determine the number of viable bacilli in the population. This technique can be used to determine the number of drug tolerant persisters in the culture. The three most critical steps in culturing biofilms are: 1) choice of an appropriate detergent-free media , closed culture container during first three weeks, and open container for subsequent incubation. Furthermore, keeping the container in a humidified condition is also critical to prevent evaporation of media during 5-week incubation. This can significantly hamper the reproducibility of the results.

Disclosures

We have nothing to disclose.

Acknowledgements

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