Tuberculosis 113 (2018) 91–98

Contents lists available at ScienceDirect

Tuberculosis

journal homepage: www.elsevier.com/locate/tube

Model Systems

Drug susceptibility testing of mature *Mycobacterium tuberculosis* H37Ra and *Mycobacterium smegmatis* biofilms with calorimetry and laser spectroscopy

Anna Solokhina[a], Gernot Bonkat[b], Ekatherina Kulchavenya[c], Olivier Braissant[a,*]

[a] Center of Biomechanics & Calorimetry Basel (COB), University Basel, C/o Department of Biomedical Engineering (DBE), Gewerbestr. 14, CH-4123, Allschwil, Switzerland
[b] Alta uro AG, Centralbahnplatz 6, CH-4051, Basel, Switzerland
[c] Urogenital Department, Novosibirsk Research TB Institution, Russia

ARTICLE INFO

Keywords:
Mycobacterium
Biofilm
Drug susceptibility test
Isothermal microcalorimetry
Tunable diode laser absorption spectroscopy

ABSTRACT

Biofilms are more resistant to antibiotics and antimicrobial stressors than planktonic bacteria; however, only a limited number of standardized assays enable investigation of this phenomenon. Here, we utilized non-invasive and independent techniques, including isothermal microcalorimetry (IMC) and tunable diode laser absorption spectroscopy (TDLAS), to measure the effect of isoniazid on metabolic activity and respiratory capability of mature *Mycobacterium tuberculosis* H37Ra (an avirulent strain) and *Mycobacterium smegmatis* biofilms. We detected only minor changes in metabolic heat production and respiratory rates (O_2 and CO_2) for mature *M. smegmatis* biofilms after antibiotic exposure. However, mature *M. tuberculosis* biofilms showed greater sensitivity to antibiotic treatment, with isoniazid exhibiting dose-dependent effects on metabolic activity and respiration. Specifically, treatment of *M. tuberculosis* biofilms with 250 µg/ml and 1 mg/ml isoniazid decreased the rate of heat production by 33% and 40%, respectively, oxygen consumption by 18% and 55%, respectively, and carbon dioxide production by 27% and 64%, respectively. These effects were prominent even after regrowth of antibiotic-treated *M. tuberculosis* H37Ra biofilms on fresh medium. Our data therefore suggest that IMC and TDLAS are appropriate for drug susceptibility testing of mature biofilms, and these techniques may facilitate study of microbial resistance to antimicrobial compounds from a bioenergetic perspective.

1. Introduction

Tuberculosis is a chronic infectious disease caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*). This organism is one of the most effective microbial killers worldwide, affecting not only the lungs, but all human organs [1]. Extrapulmonary tuberculosis involves a broad spectrum of mycobacterial infections, where the pathogen can spread outside the lungs and infect tissues such as the lymph nodes, genitourinary tract, bones, joints, spine, and gastrointestinal tract [2]. Although the diagnostic methods available for *Mtb* have significantly improved in recent years, drug susceptibility testing and treatment remain complicated. The conventional antimycobacterial treatment (RIPE: Rifampicin, Isoniazid, Pyrazinamid, Ethabutol) is only partially effective [1], and in many cases, lack of effectiveness of antibiotic treatments is associated with the presence of bacterial genetic determinants (e.g., resistance genes) [3]. In addition, biofilm formation has been shown to provide protection against therapeutic regimens. *Mtb* biofilms (or biofilm-like structures) have been observed in primary lesions associated with residual necrosis or as a coating in cavities [4,5]. Extracellular polymeric substances (EPS) produced by mycobacteria within a biofilm limit drug diffusion and thus protect individual cells from antimicrobial activity [5–7]. Consequently, mycobacterial biofilm infections are difficult to treat, leading to frequent relapse of disease [8]. Intriguingly, recent studies have also indicated that *Mtb* biofilms may exhibit higher metabolic rates than initially hypothesized [9].

In this context, the ability to perform drug susceptibility testing on *Mtb* biofilms is of primary importance. However, this task remains difficult. Many *in vitro* assays have been developed to study biofilm growth, structure, and physiology, and some of these allow for the screening of antibiotics to assess their activity against cells within a biofilm. Semi-automated methods for biofilm antibiotic susceptibility testing are often based on determination of minimum biofilm eradication concentration (MBEC) or minimum biofilm inhibition concentration (MBIC) [10] These assays utilize 96-well plates covered by a plastic lid containing 96 pegs on which biofilms can develop and eventually be

* Corresponding author.
E-mail addresses: a.solokhina@googlemail.com (A. Solokhina), Bonkat@alta-uro.com (G. Bonkat), olivier.braissant@unibas.ch (O. Braissant).

https://doi.org/10.1016/j.tube.2018.09.010
Received 19 March 2018; Received in revised form 28 September 2018; Accepted 29 September 2018

transferred to a solution with antimicrobial compounds to be tested. Unfortunately, to save time, these assays are often conducted early during biofilm development (right after adhesion), and none can be used to assess mature biofilms (more than 4–5 weeks old). Furthermore, MBEC assays are performed using a liquid solution surrounding the plastic peg covered with a thin biofilm. With such a large liquid phase in contact with a thin biofilm, diffusion of drugs is rather fast, and measured minimum inhibitory concentrations (MICs) may be underestimated. Finally for thicker biofilm, several other technical limitations such as poor dye penetration and biofilm heterogeneity make assays difficult [6,11] In this context, label free and non-destructive methods could be helpful (see below).

Biofilms differ from their planktonic cell counterparts in many ways, from growth rate to surface protein expression, and mycobacteria are no exception. However, due to their slow growth rate, biofilms of *Mtb* and mycobacteria other than *M. tuberculosis* (MOTT) have not been extensively studied, particularly with respect to drug susceptibility. The use of polystyrene bottles for growth of mycobacterial biofilms is quite practical and allows for the investigation of drug tolerance in these bacteria [8] Unfortunately, these biofilms can be used only once, and therefore it is impossible to make direct comparisons of biofilm growth or metabolic activity before, during, and after antibiotic treatment for the same biofilm. This is further complicated by the fact that biofilms have been shown to be heterogeneous in both structure and activity [6,12] Indeed, to the best of our knowledge, there are no studies that have described the analysis of drug susceptibility testing using the same undisturbed mature mycobacterial biofilms. To address these issues, we have developed a method for growing biofilms on nylon filters, which allow simple and easy transfer from one medium to another. However, label-free, non-invasive and non-destructive measurement techniques are also required so that the same biofilm can be used several times and transferred to different conditions (e.g., different antibiotics).

Appropriate antibiotics and associated concentrations for treatment of clinical infection are usually chosen based on drug susceptibility testing, which is performed on bacterial cultures that have been isolated from a decontaminated sample (mostly sputum). However, because these assays mostly rely on liquid cultures, they can only measure the behavior of planktonic cells. To assess the drug susceptibility of mycobacterial biofilms, which may be more relevant in vivo, other techniques are needed. In this context, isothermal microcalorimetry (IMC) represents a promising tool. IMC can be used to measure an organism's metabolic heat production in the μW or sub-μW ranges; one active bacterium releases approximately 2 pW of thermal power [13,14] Heat production rate is proportional to the metabolic activity of cells within a biofilm. Therefore, changes in metabolic activity associated with drug treatment efficiency can be directly correlated with IMC measurements. In addition, IMC is a label-free technique that allows processing of specimens on solid media and leaves samples undisturbed. Here, we utilized this assay to measure the same undisturbed model biofilm, before and after exposure to antimycobacterial compounds, and additionally, to follow changes in metabolic activity during exposure to antimicrobials.

Tunable diode laser absorption spectroscopy (TDLAS) is another independent and non-invasive method that can be used to analyze the impact of antibiotics on mature mycobacterial biofilms. This laser spectroscopy technique measures gas concentrations (O_2 and CO_2) in the headspace of the tested glass vials [9,15] Because mycobacteria are aerobic microorganisms that consume oxygen and release carbon dioxide, TDLAS can be used to follow respiration rates from mature biofilms being exposed to different conditions and stressors. It has previously been shown that respiration rates measured with TDLAS are in close agreement with the metabolic heat production recorded by IMC [9].

In this study, we used IMC and TDLAS to develop a continuous method for measuring the metabolic activity of *Mtb* and *M. smegmatis* biofilms grown on nylon filters in response to antimicrobial treatment. As proof of concept, we further show that these techniques can be effectively used to measure the susceptibility of model mature mycobacterial biofilms of both *M. smegmatis* and *Mtb* to isoniazid, a commonly used anti-tuberculosis drug. Metabolic activities were assessed before, during, and after antibiotic exposure and compared using IMC and TDLAS, and our data suggest that these techniques can be effectively utilized for drug susceptibility testing of mature mycobacterial biofilms.

2. Materials and methods

2.1. Instrumentations

In this study, we utilized IMC (TAM Air, Waters/TA Instruments, USA) and TDLAS (WILCOMAT Instruments, Wohlen, Switzerland) to analyze the effect of INH on metabolic activity (e.g., heat flow as a metric for metabolic heat production), growth (μ), lag phase (λ), and respiration activity of mature mycobacterial biofilms.

2.2. Bacterial culture and biofilm preparation

Precultures of *Mycobacterium* strains were prepared in liquid Middlebrook 7H9 medium, containing 10% oleic acid, albumin, dextrose, and catalase (OADC) enrichment solution and incubated at 37 °C (between 2 and 3 days for *M. smegmatis* (DSM 43465) and up to 14 days for *Mtb* H37Ra (ATCC 25177, an avirulent strain). After sufficient growth was obtained, corresponding to an optical density at 600 nm (OD_{600}) of 0.25–0.5, the liquid mycobacterial culture to be tested was spread on nylon filters (0.2 μm, Millipore, Burlington MA, USA), which were previously cut to fit into 20 ml IMC vials, using 10 μl inoculating loops. After inoculation, the filters were placed on a Petri dish containing fresh 7H9 agar medium with 10% OADC (1.5% agar final concentration). *Mycobacterium* biofilm formed on these filters were transferred to fresh 7H9 agar until a mature biofilm state was achieved (8–10 days for *M. smegmatis* and 30–35 days for *Mtb*); biofilms were transferred to fresh medium every 7–10 days. Biofilms were considered mature when no visible changes could be seen in their size or appearance. The nylon membrane surface covered by biofilm was ca 200 mm2 when size did not increase anymore (surface coverage was measured by image analysis using ImageJ – the biofilms which had the closest surface were selected for future experiments). At this point we assumed that a “steady state” was reached, as the biofilm was not experiencing further expansion (i.e., no biomass increase was visible). Consequently, we could safely assume that any changes detected in metabolic heat production were due to changes in metabolic activity induced by antimicrobial treatment. All media and materials (including the cut nylon filters) were autoclaved for 20 min at 120 °C.

2.3. Determining metabolic activity in biofilm using isothermal microcalorimetry

To detect biofilm heat production with IMC, mature mycobacterial biofilms grown on nylon filters were placed in 20 ml calorimetry glass vials (Waters/TA Instruments) containing slanted solid 7H9 medium supplemented with 10% OADC (Fig. 1). After sample introduction, the TAM Air (Waters/TA Instruments) was set at a temperature of 37 °C ± 0.01 °C. This device has eight measuring channels and eight slots for inert thermal references of the same heat capacity and conductivity as the samples (in this study, water agar slants of the same volume were prepared as inert thermal references). The heat flow was measured differentially between sample and reference for each sample continuously in real time. As soon as no metabolic heat production could be observed (corresponding to the total consumption of O_2 and to a partial exhaustion of resources), the same undisturbed filters were passaged to fresh solid 7H9 medium containing two different concentrations of INH (250 μg/ml and 1 mg/ml), and metabolic activity