



Article

Reductive Stress-Induced Biofilm Formation Mediates Mycobacterial Resistance to Macrophage Capture and Nitric Oxide Exposure

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Abstract

Mycobacteria form cellulose-containing biofilms and exhibit drug resistance at infection sites. However, the function of mycobacterial biofilms in host defense remains unclear. Herein, we demonstrate that reductive stress-induced mycobacterial biofilms evade macrophage capture and protect bacilli from nitric oxide stress. We first determined the optimal conditions for biofilm formation by mycobacteria. Using green fluorescent protein (GFP)-labeled mycobacteria, we then examined the protective effect of biofilms on the capture of bacilli by macrophages and found that macrophage capture was inhibited in the presence of biofilms. Furthermore, we constructed GFP-expressing mycobacteria that respond to acidic pH and nitric oxide stress, both of which are bactericidal factors. The results showed that mycobacterial biofilms protect bacilli from nitric oxide-mediated stress, but not from acidification. Finally, the removal of biofilms by cellulase enhanced the capture of mycobacteria by macrophages and the exposure of mycobacteria to nitric oxide. These findings highlight the protective roles of mycobacterial biofilms in innate immunity, particularly against macrophage capture and nitric oxide-induced stress.

Keywords: mycobacteria; biofilm; reductive stress; cellulase; nitric oxide

1. Introduction

Biofilms are composed of polysaccharides, proteins, nucleic acids, and lipids, which determine their properties [1,2]. Biofilms form on almost all surfaces of environmental objects and artifacts, including medical devices, and play a crucial role in the persistence and pathogenesis of bacterial infections. Many bacteria have evolved the ability to form biofilms as a survival strategy in detrimental environments [3]. These biofilms act as protective barriers, shielding the bacilli from harmful substances, such as antimicrobials and host defenses, and facilitating bacterial communication through the exchange of metabolites and genetic material [2,4].

In clinical settings, bacteria form biofilms on artifacts, providing a scaffold for bacterial growth and conferring drug resistance, making drug treatment difficult [5,6]. Nontuberculous mycobacteria (NTM), which mainly reside in the natural environment [7,8], can also form biofilms on indwelling medical devices and lead to persistent infections [9]. One study reproduced a mouse model demonstrating biofilm formation by NTMs around



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indwelling catheters [10]. Interestingly, *Mycobacterium tuberculosis* (Mtb), which is transmitted via human-to-human infection, has been reported to form cellulose-containing biofilms around bacilli in the lungs following infection, even in the absence of foreign materials [11]. Bacterial biofilm formation is affected by various environmental factors, such as temperature, pH, oxygen, oxidative stress, and nutrients; however, mycobacteria form cellulose-containing biofilms in response to reductive stimuli [12–14]. Albumin is required for mycobacterial biofilm formation following reductive stimuli; this trigger is likely broadly conserved among mycobacterial species [15,16]. Previous reports have shown that biofilms play a role in conferring drug resistance in mycobacteria; however, this biofilm is degraded by treatment with cellulase from *Trichoderma viride*, which digests cellulose [11,15]. Moreover, cellulase-overexpressing Mtb strains were removed more easily from murine lungs than the wild-type strain at an early stage of infection [11], suggesting that mycobacterial biofilms may possess protective functions against innate immunity in vivo. Several types of bacterial biofilms have been proposed to protect against innate immunity [17–19]. In innate immune responses against mycobacteria, macrophages capture mycobacteria as sentinels and attempt to kill them through phagolysosomal acidification [20,21] and the induction of nitric oxide (NO) production [22,23]; however, the protective function of mycobacterial biofilms against these immune responses has not been elucidated.

In studies exploring host–mycobacterial interactions, fluorescent proteins are frequently employed in conjunction with mycobacterial promoters to monitor bacterial conditions and localization, which can be detected using flow cytometry or fluorescence microscopy. Plasmids encoding fluorescent proteins under mycobacterial promoters for constitutive expression have been developed to monitor bacterial responses to extracellular stimuli [24]. To evaluate localization or phagocytosis, the *smyc* promoter is commonly used to achieve constitutive expression of proteins [25]. The *aprA* gene encodes a membrane-associated regulatory protein that is induced under acidic conditions via the PhoPR system and modulates the lipid composition of the cell surface and intracellular adaptation to the macrophage phagosomal environment [26]; the promoter of *aprA* is activated by acidic pH stress. Meanwhile, the promoter of *icl1* is activated by NO stress in mycobacteria. The *icl1* gene encodes the isocitrate lyase, which enables Mtb to persist during infection by supporting fatty acid metabolism and allowing bacterial survival in activated macrophages, suppressing the interferon (IFN)- γ -induced immune response [27,28]. However, these fluorescent tools have not been applied to the functional analysis of mycobacterial biofilms.

In this study, we aimed to investigate the protective functions of mycobacterial biofilms against host defenses such as macrophage capture, acidic pH, and NO using *Mycobacterium smegmatis* (Msm) and *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), which are used to understand common principles in mycobacteria. We established mycobacterial strains that constitutively expressed green fluorescent protein (GFP) to assess macrophage capture. The rate of bacterial phagocytosis by macrophages decreased in the presence of biofilm. An assay using acidic pH- and NO-inducible GFP-expressing mycobacteria showed that the biofilm prevented NO exposure of mycobacteria, but not at acidification. Mycobacterial biofilms were susceptible to cellulases derived from *Aspergillus niger* and *Trichoderma viride* in addition to mycobacterial cellulase Rv0062. Cellulase treatment impairs the protective functions of mycobacterial biofilms against macrophage capture and NO exposure. Our results revealed that mycobacterial biofilms function to protect the bacteria from host defenses, and that biofilm removal improves the effectiveness of these immune responses.

2. Materials and Methods

2.1. Mycobacterial Strains, Culture, and Handling

M. smegmatis mc²-155, *M. bovis* BCG Tokyo 172, and *M. tuberculosis* Erdman were used as the parental strains for all in vitro and macrophage infection experiments. For growth, these strains were grown at 37 °C under shaking (150 rpm) in 7H9 medium containing Middlebrook 7H9 medium (BD Difco, 271310, Franklin Lakes, NJ, USA) supplemented with 0.2% glycerol, 0.05% Tween-80, and Middlebrook oleic acid–albumin–dextrose–catalase (OADC) enrichment (BD BBL™, 212240, Franklin Lakes, NJ, USA) or on 7H10 agar containing Middlebrook 7H10 (BD Difco, 262710, Franklin Lakes, NJ, USA) supplemented with 0.5% glycerol and OADC enrichment. For flow cytometry-based assays, samples were fixed with 4% paraformaldehyde (FUJIFILM Wako Pure Chemical Corporation, 163-20145, Osaka, Japan), and GFP fluorescence was measured using a BD FACSCanto II (BD; Becton, Dickinson and Company, Franklin Lakes, NJ, USA). For plate-based fluorescence or absorbance measurements, a NIVO™ plate reader (Revvity, Waltham, MA, USA) was used. Flow cytometry data were analyzed using FlowJo (v10.8.1; Tree Star, Inc., Ashland, OR, USA). All studies involving virulent *M. tuberculosis* were performed in a biosafety level 3 (BSL-3) facility according to the institutional biosafety guidelines of the National Institute of Infectious Diseases.

2.2. Analysis of Mycobacterial Biofilms

2.2.1. Mycobacterial Biofilm Formation

For biofilm formation, bacteria were cultured in 7H9 medium containing 0.2% glycerol, 0.05% Tween 80, and 5% OADC and aerated with shaking (150 rpm) at 37 °C. These mycobacteria were grown to the early logarithmic phase ($OD_{600} = 0.2–0.4$) and inoculated at a volume of 250 µL per well in 24-well plates or 50 µL per well in 96-well plates. The cultures were then exposed to the same volume of 2, 6, or 20 mM reduced dithiothreitol (DTT) (FUJIFILM Wako Pure Chemical Corporation, 042-29222, Osaka, Japan) or 10, 30, or 100 mM glutathione (GSH) (FUJIFILM Wako Pure Chemical Corporation, 073-02013, Osaka, Japan) in 7H9 medium only without glycerol, Tween-80, or OADC. The samples were then incubated without shaking for 24 h to form adherent biofilms.

As shown in Figure S1B, OADC was prepared by mixing 50 mg oleic acid (Sigma-Aldrich, O1008, St. Louis, MO, USA), 5 g bovine albumin (FUJIFILM Wako Pure Chemical Corporation, 019-27051, Osaka, Japan), 4 mg catalase (Sigma-Aldrich, C40, St. Louis, MO, USA), and 2 g dextrose (FUJIFILM Wako Pure Chemical Corporation, 047-31165, Osaka, Japan) in 100 mL of deionized distilled water (DDW). ODC was prepared by mixing oleic acid, dextrose, and catalase in 100 mL DDW at the above concentrations. Mycobacteria at the early logarithmic phase ($OD_{600} = 0.2–0.4$) were washed with 7H9 medium containing 0.2% glycerol, 0.05% Tween-80 and adjusted to the same volume. Next, 50 µL of the reconstituted culture was added to 96-well plates and exposed to the same volume of 20 mM reduced DTT in DDW or two-fold concentrations of OADC and ODC.

2.2.2. Crystal Violet (CV) Staining Assay of Mycobacterial Biofilms

This assay was used to quantify biofilm biomass. The CV assay was performed in a 96-well tissue culture plate (AS ONE Corporation, 1-1601-04-99, Osaka, Japan). After biofilm formation, the medium above the biofilm surface was gently removed, and then the biofilm was dried for 2 h at 80 °C. Next, 200 µL of 1% CV (Sigma-Aldrich, V5265, St. Louis, MO, USA) was carefully added to each well and incubated for 30 min at room temperature. Afterward, the CV reagent was removed, and the mycobacterial biofilms were gently washed four times with DDW to avoid biofilm dispersal.

CV bound to the biofilm was then extracted by incubation with 200 µL of 95% ethanol for 10 min, and the supernatants were collected in a new 96-well plate and diluted 20–40 fold with 95% ethanol. The absorbance of the extracted CV was measured at 590 nm using a NIVO™ plate reader.

2.3. Exposure of Mycobacteria to Acidic, Oxidative, or Temperature Stress

To assess the effect of hypoxic stress on mycobacterial biofilm formation, cultures were incubated under atmospheric, microaerophilic (MITSUBISHI GAS CHEMICAL Inc., A-28, Tokyo, Japan), or hypoxic (MITSUBISHI GAS CHEMICAL Inc., A-03, Tokyo, Japan) conditions during DTT stimulation for 1 day.

To examine the effect of temperature changes on biofilm formation, mycobacteria were stimulated with or without 10 mM DTT at 4 °C, 16 °C, 25 °C, 32 °C, or 37 °C for 1 day. In addition to incubation at stable temperature conditions, the incubation temperature was changed in some setups as follows: 37 °C (1 day) and then to 4 °C (3 days) or 4 °C (1 day) to 37 °C (3 days).

For assessment of the effect of acidic stress on biofilm formation, mycobacteria were cultured in 7H9 medium adjusted to pH 4.0, 5.0, 6.7, 8.0, 9.0 with or without 10 mM DTT for 1 day.

For assessment of the effect of oxidative stress on biofilm formation, mycobacteria were cultured in 7H9 medium with or without 10 mM DTT at 0.1 and 1 mM hydrogen peroxide (H₂O₂) (FUJIFILM Wako Pure Chemical Corporation, 081-04215, Osaka, Japan) or 0.1 and 1 mM NO (Cayman Chemicals, NO donor DETA NONOate, 82120, Ann Arbor, MI, USA) for 1 day.

2.4. Plasmid Constructs and Bacterial Transformation

2.4.1. Design of GFP-Expressing Plasmids and Transformation into *E. coli*

The pYM301a plasmid was used as the backbone vector, which is an *Escherichia coli*-*Mycobacterium* shuttle vector that carries the *hsp60* promoter, integrase gene, and a hygromycin resistance gene [29]. Details of the plasmids and primers used are shown in the Supplemental Tables S1 and S2. The pYM301a-*smyc*-sfGFP plasmid was constructed by fusing the pYM301a vector segment, which was amplified using the primers pYM301a-F and pYM301a-R, the *smyc* promoter, which was amplified using the primers P_{*smyc*}-F and P_{*smyc*}-R, and the sfGFP gene, which was amplified using the primers sfGFP-F and sfGFP-R.

To construct pYM301a-*aprA*-sfGFP or pYM301a-*icl1*-sfGFP, pYM301a-*smyc*-sfGFP was amplified using the primers sfGFP-F and pYM301a-R. The *aprA* promoter was amplified using the primers P_{*aprA*}-F and P_{*aprA*}-R, while the *icl1* promoter was amplified from the genomic DNA of *M. tuberculosis* using the primers P_{*icl1*}-F and P_{*icl1*}-R.

These amplified segments were fused using an In-Fusion cloning system (Takara Bio Inc., Z9650N, Kusatsu, Shiga, Japan), and the resulting fragments were introduced into competent *E. coli* DH5α cells (NIPPON GENE Co., Ltd., 314-06234, Tokyo, Japan) by heat shock for 30 s at 42 °C. After colony growth in LB medium supplemented with 150 µg/mL hygromycin B (FUJIFILM Wako Pure Chemical Corporation, 085-06153, Osaka, Japan), the plasmids were collected using a miniprep kit (New England Biolabs, T1110L, Ipswich, MA, USA).

2.4.2. Transformation into Mycobacteria

To establish mycobacteria with constitutive GFP expression, *M. smegmatis* mc²-155 or *M. bovis* BCG in the middle of the logarithmic growth phase (OD₆₀₀ = 0.6–0.8) were harvested and concentrated to 1/50 of their volume after washing three times with 10% glycerol. Cells were then electroporated with 1 µg of pYM301a-*smyc*-sfGFP plasmid (quantified using a Nanodrop spectrophotometer) per 100 µL of bacterial suspension in 10% glycerol in

a 0.1 mm cuvette. Electroporation was performed under the following conditions: 1500 V, 25 μ F, 200 Ω using Gene Pulser X cell (BIO-RAD, Hercules, CA, USA).

After the mycobacteria recovered in 7H9 medium supplemented with 0.2% glycerol, 0.05% Tween-80 and 10% OADC overnight, the transfectants were selected on 7H10 agar plates containing 100 μ g/mL hygromycin for *M. smegmatis* or 50 μ g/mL for *M. bovis* BCG, and incubated for 3 or 14 days, respectively, until colonies formed. Colonies were picked up and cultured in 7H9 medium until the middle of the logarithmic phase and stored at -80°C until further analysis. The bacterial concentration (CFU) of the stock was determined by serial dilution on 7H10 agar.

To establish acid- or NO-inducible GFP-expressing mycobacteria, transformation was performed with the pYM301a-*aprA*-sfGFP or pYM301a-*icl1*-sfGFP plasmids, respectively, using the electroporation method described above. Transformants were selected on 7H10 agar plates containing hygromycin. All recombination experiments were approved by our institute.

2.4.3. Design of Mycobacterial Cellulase-Expressing Plasmids and Protein Expression

These plasmids were constructed by inserting either the Rv0062-His-tag or glutathione S-transferase (GST)-tag-Rv1090-His-tag fragments into the pET49b vector using the In-Fusion cloning system. The pET49b plasmid was amplified using the primers pET49b-F and pET49b-R. Rv0062-His-tag and Rv1090-His-tag were cloned from the chromosomal DNA of *M. tuberculosis* Erdman using the primer pairs Rv0062-F/Rv0062-His-R and Rv1090-F/Rv1090-His-R, respectively. GST-tag was cloned from the pCold plasmid (Takara Bio Inc., 3364, Kusatsu, Shiga, Japan) using the primers GST-F/GST-R.

After the transformation of these plasmids into the *E. coli* Rosetta 2 cells, successful transformants were selected on LB agar containing 50 μ g/mL kanamycin (FUJIFILM Wako Pure Chemical Corporation, 113-00343, Osaka, Japan) and 34 μ g/mL chloramphenicol (FUJIFILM Wako Pure Chemical Corporation, 036-10571, Osaka, Japan). The selected colonies were then cultured overnight in LB medium containing the same antibiotics, and 1 mL of each overnight subculture was inoculated into 100 mL of LB medium. After incubation for 3 h ($\text{OD}_{600} = 0.4\text{--}0.5$), protein expression was induced by adding 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) (FUJIFILM Wako Pure Chemical Corporation, 096-05143, Osaka, Japan) and subsequent culture for another 3 h at 37°C . The cells were then harvested by centrifugation (6000 rpm, 5 min, room temperature), and the cell pellet was lysed according to the manufacturer's protocol (ATTO Corporation, WSE-7423, Tokyo, Japan). The supernatant was then incubated with cobalt affinity beads (Takara Bio, Z5501N) for 1 h with shaking at room temperature. The beads were washed three times with a buffer containing 25 mM Tris-HCl and 200 mM NaCl, and the target proteins were eluted with 4 mL of a 250 mM imidazole buffer. The eluted proteins were concentrated using a 10-kDa cutoff ultrafiltration system (3000 rpm, 1 h, 4°C ; Merck, Amicon® Ultra-4 mL, Darmstadt, Germany) and washed three times with 10 mM Tris buffer (pH 8.0) (NIPPON GENE Co., Ltd., 314-90065, Tokyo, Japan) and 250 mM NaCl (FUJIFILM Wako Pure Chemical Corporation, 195-01663, Osaka, Japan). The purity of the proteins was assessed using Coomassie Brilliant Blue staining, and protein concentrations were measured using the BCA assay (Thermo Fisher Scientific, 23225, Waltham, MA, USA).

2.5. Capture of GFP-Labeled Mycobacteria by Macrophages

(1) Target preparation

Biofilm-forming group: GFP-labeled *M. smegmatis* and *M. bovis* BCG were induced to form biofilms as described above. After induction, the culture supernatant was removed, and the wells were gently washed with antibiotic-free Dulbecco's Modified Eagle Medium

(DMEM) (Sigma-Aldrich, D6546-500ML, St. Louis, MO, USA) containing 2% fetal calf serum (FCS) (Sigma-Aldrich, 17012-500ML, St. Louis, MO, USA).

Non-biofilm (control) group: GFP-labeled mycobacteria were cultured without biofilm induction. Following supernatant removal, the wells were replenished with DMEM with 2% FCS. The planktonic bacterial cells were allowed to sediment for at least 4 h. This step ensured that the bacterial concentration at the bottom of the wells was comparable to that of the biofilm-forming group.

(2) Co-culture of mycobacteria and macrophages

RAW264.7 macrophages (10^5 cells/200 μ L of 10% FCS containing antibiotic-free DMEM) were then added to each prepared well and incubated for 4 h. After incubation, the supernatant was removed and the RAW 264.7 cells were harvested using 2 mM EDTA/PBS with gentle pipetting. GFP-positive macrophages were then analyzed using a BD FACS Canto-II flow cytometer. To confirm comparable bacterial inputs between planktonic and biofilm conditions, CFU were determined from washed wells.

2.6. Evaluation of Cellulase Activity and Cellulase Treatment of Biofilms

2.6.1. Carboxymethyl Cellulose (CMC) Agar and Congo Red Staining

In this experiment, 1.5% agar plates containing 1% CMC (Sigma-Aldrich, 419273, St. Louis, MO, USA) were prepared to evaluate cellulase activity. Briefly, 5 μ L of 1 mg/mL cellulase was spotted onto the agar plates and incubated at 37 °C for 1 h. After incubation, the plate surface was gently washed twice with 10 mL of DDW. Next, 10 mL of 0.1% Congo red (FUJIFILM Wako Pure Chemical Corporation, 039-23192, Osaka, Japan) reagent was added to the plate and incubated for 15 min at room temperature without shaking. For decolorization, the plate was washed with 20 mL of fresh DDW every 5 min for 30 min.

2.6.2. Inhibition of Mycobacterial Biofilm Formation Using Cellulases

Mycobacterial biofilms were treated with cellulase at the same time that DTT was added and incubated for 24 h. The resulting biofilms were then treated with cellulases derived from *T. viride* (Merck, 219466, Darmstadt, Germany), *T. reesei* (Sigma-Aldrich, C2730, MO, USA), or *A. niger* (Sigma-Aldrich, C1184, St. Louis, MO, USA) and the produced mycobacterial cellulases, Rv0062-His-tag and GST-tag-Rv1090-His-tag.

2.7. Exposure of Mycobacteria to Acidic and Nitric Oxide Stress and Detection of GFP Signals

To examine the protective effect of mycobacterial biofilms against nitric oxide stress, *M. smegmatis* and *M. bovis* BCG were cultured in 7H9 medium supplemented with OADC until the early logarithmic phase ($OD_{600} = 0.2\text{--}0.4$), and 250 μ L of the culture was inoculated into each well of a 24-well plate. An additional 250 μ L of 7H9 medium with or without 20 mM DTT was added to each well and incubated for 1 day. After biofilm formation, the biofilms were gently washed with 7H9 medium to remove the DTT. Then, 500 μ L of 7H9 medium including 0.1 mM nitric oxide was added to each bacterial culture well and incubated at 37 °C for 2 days under mild shaking (100 rpm). GFP expression in the wells was detected by NIVO™ plate reader.

To examine the protective effect of mycobacterial biofilms against acidic stress, Biofilms of the culture of *M. smegmatis* and *M. bovis* BCG were induced as above assay. After biofilm formation, the biofilms were gently washed with 7H9 medium to remove the DTT. Then, 500 μ L of 7H9 medium adjusted to pH 5.5 was added to each bacterial culture well and incubated at 37 °C for 1 day. GFP expression in the wells was detected by a NIVO™ plate reader.

2.8. CFU Measurements

To quantify bacterial survival after exposure to acidic or nitric oxide stress in the presence or absence of biofilm, both planktonic and biofilm-associated bacteria were recovered from each well of a 24-well plate. Biofilms were detached by vigorous pipetting and scraping of the well surface, and the suspension was homogenized in PBS containing 0.05% Tween-80 to minimize clumping. Homogenized samples were 10-fold serially diluted and 150 µL of appropriate dilutions were plated onto Middlebrook 7H10 agar plates. Colonies were counted, and CFU were calculated as CFU per well (or CFU/mL) based on the dilution factor and plated volume. Plates were incubated at 37 °C for 3–5 days (*M. smegmatis*) or 17–24 days (*M. bovis* BCG) before counting.

2.9. Scanning Electron Microscopy (SEM) Analysis

M. bovis BCG was grown in 7H9 medium supplemented with 0.2% glycerol and 10% OADC without Tween-80 until it reached the logarithmic growth phase, and the cells were diluted to an OD₆₀₀ = 0.3 with the same medium. DTT was then added to the bacterial suspension at a final concentration of 10 mM. The test materials were cut into 6 × 8 × 2 mm pieces (width × length × thickness; Standard Test Piece, Inc., Vantaa, Finland), cleaned with 70% ethanol, and sterilized under UV light for 20 min. The sterilized pieces were placed at the bottom of a 24-well plate, and the bacterial cultures were added. The samples were then cultured at 37 °C under atmospheric conditions for 14 days.

Biofilms were ultrastructurally analyzed by field emission SEM (FE-SEM) (SU8600, Hitachi High-Tech, Tokyo, Japan) as follows. Briefly, the biofilms were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in PBS overnight at 4 °C, dehydrated in a graded ethanol series (50–100%) at room temperature, and dried in a critical point dryer using CO₂ (CPD300, Leica Microsystems, Wetzlar, Germany). The samples were then coated with osmium using an osmium plasma coater (Neoc-Pro/P; Meiwafoysis, Tokyo, Japan) and visualized using FE-SEM at an accelerating voltage of 1 kV.

2.10. Statistical Analysis

The flux values of the fluorescent intensities were reported as the mean ± SD. The significance of differences was determined using one-way ANOVA with Tukey's test for multiple comparisons and Student's *t*-test using GraphPad Prism 10 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Reductive Stimuli Induce Biofilm Formation in *M. smegmatis* and *M. bovis* BCG

To elucidate the protective functions of mycobacterial biofilms against host defense, Msm and BCG were used. We first verified whether these mycobacteria possessed the ability to form biofilms in vitro using CV staining. Our results demonstrated that both Msm and BCG formed biofilms, depending on the concentrations of DTT and GSH used as reductive stimuli (Figures 1A and S1A). To identify the optimal conditions for biofilm formation, we investigated whether other environmental factors, such as oxygen concentration, temperature, oxidative stress, and pH stress, affect mycobacterial biofilm formation. Our findings indicate that oxygen is essential for mycobacterial biofilm formation, as biofilms develop in an oxygen concentration-dependent manner (Figure 1B). However, shifts in oxygen concentration from hypoxic to atmospheric levels or vice versa did not trigger biofilm formation (Figure 1C). In terms of temperature, biofilm formation was more efficient at 37 °C under reductive stress rather than 4 °C, 16 °C, 25 °C, or 32 °C (Figure 2A). Moreover, an increase in temperature (from 4 °C to 37 °C) promoted reductive stress-induced biofilm formation, whereas a decrease in temperature (from 37 °C to 4 °C)

inhibited it (Figure 2B). In contrast, temperature shifts alone, in the absence of reductive stress, did not affect mycobacterial biofilm formation (Figure 2B). We further examined whether pH stress, an important factor inhibiting mycobacterial growth, affected biofilm formation. DTT-induced biofilm formation occurred over a broad pH range, whereas it was partially inhibited at acidic or alkaline pH (Figure 3A). Oxidative stress, such as that induced by H_2O_2 and NO, did not induce biofilm formation, nor did it inhibit DTT-induced biofilm formation (Figure 3B). These results suggested that changes in pH or oxidative stress do not trigger mycobacterial biofilm formation. We then compared biofilm formation by varying the types of nutrients supplemented in the culture medium (the composition of each medium is indicated in the legend of Figure S1). The results showed that these mycobacteria required albumin for biofilm formation (Figure S1B) and each biofilm formation was inhibited by cellulase treatment (Figure S1C). The properties of biofilms formed by these non-pathogenic mycobacteria were also observed in Mtb biofilms, which are pathogenic to humans (Figure S1A–C). These results confirmed that reductive stimuli and albumin are required for biofilm formation by Msm, BCG, and Mtb, indicating that the basic properties of biofilm formation are conserved irrespective of differences in virulence among these strains.

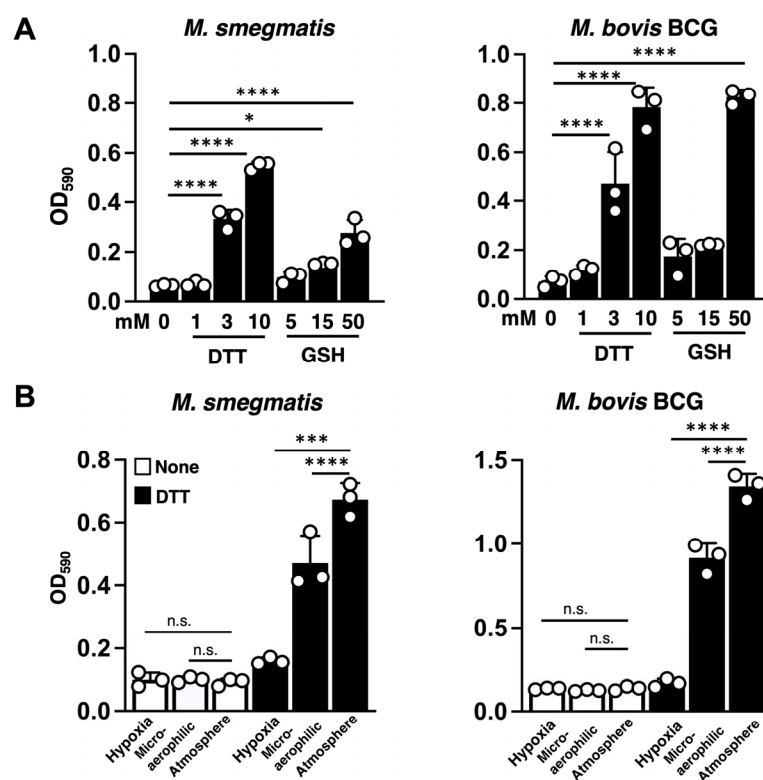


Figure 1. Cont.

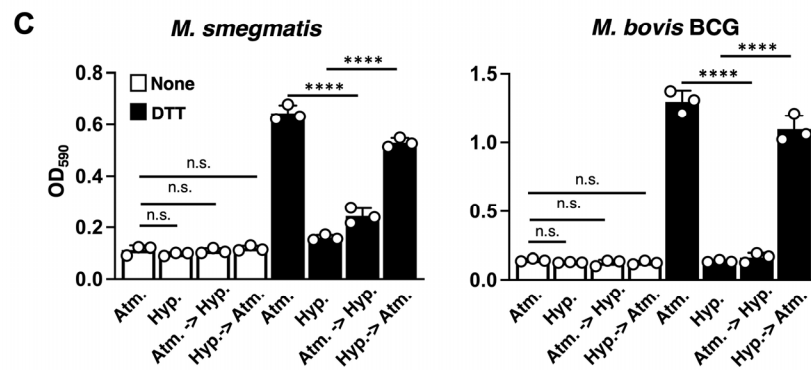


Figure 1. Analysis of reductive stimuli and oxygen concentrations required for the formation of mycobacterial biofilms. (A) Effects of reductive stress on biofilm formation. Crystal violet staining assays of Msm and BCG exposed to DTT (1, 3, and 10 mM) and GSH (5, 15, and 50 mM). (B) Effects of oxygen on mycobacterial biofilm formation. Msm and BCG were stimulated with or without DTT (10 mM) under hypoxic, microaerophilic, and atmospheric conditions. (C) Effect of oxygen concentration on mycobacterial biofilm formation. Msm and BCG were stimulated with or without DTT (10 mM) under hypoxic conditions (Hyp), atmospheric conditions (Atm), hypoxic to atmospheric conditions, or atmospheric to hypoxic conditions. All experiments were performed in triplicate, and each experiment was performed twice. Statistical analysis was performed using one-way ANOVA test and Tukey's test for multiple comparisons: * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$. n.s., not significant.

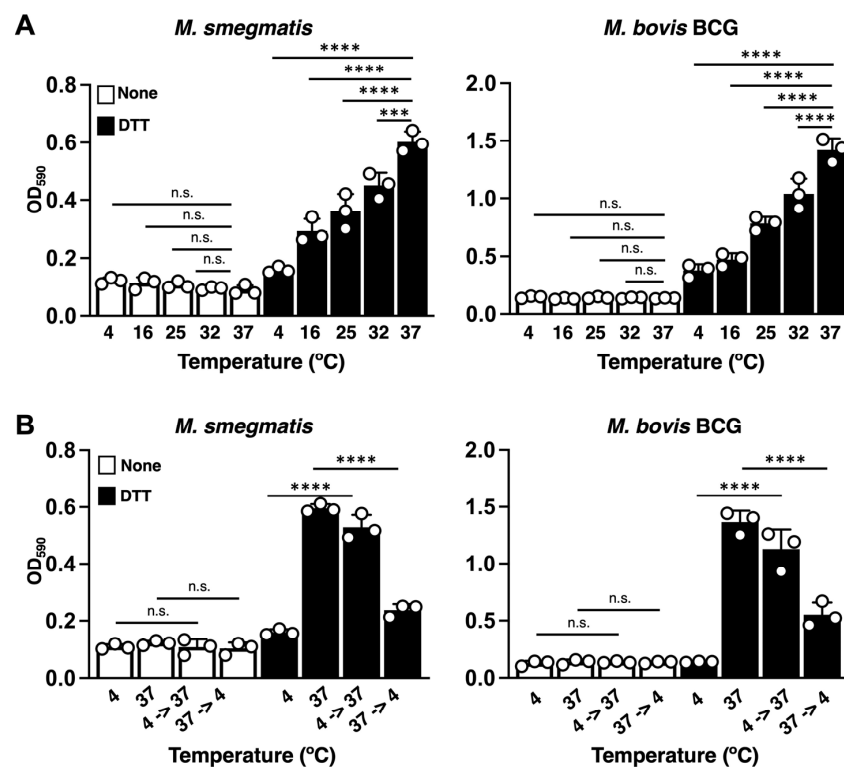


Figure 2. Analysis of the optimal temperature for mycobacterial biofilm formation. (A) Msm cells were stimulated with or without DTT (10 mM) at 4 °C, 16 °C, 25 °C, 32 °C or 37 °C, as well as under temperature-changing conditions from 37 °C (1 day) to 4 °C (3 days) and 4 °C (1 day) to 37 °C (3 days). (B) BCG were stimulated with or without DTT (10 mM) at 4 °C, 16 °C, 25 °C, 32 °C, or 37 °C, as well as under temperature-changing conditions from 37 °C (1 day) to 4 °C (3 days) and 4 °C (1 day) to 37 °C (3 days). All experiments were performed in triplicate, and each experiment was performed at least twice. Statistical analysis was performed using one-way analysis of variance and Tukey's test for multiple comparisons: *** $p < 0.001$, **** $p < 0.0001$. n.s., not significant.

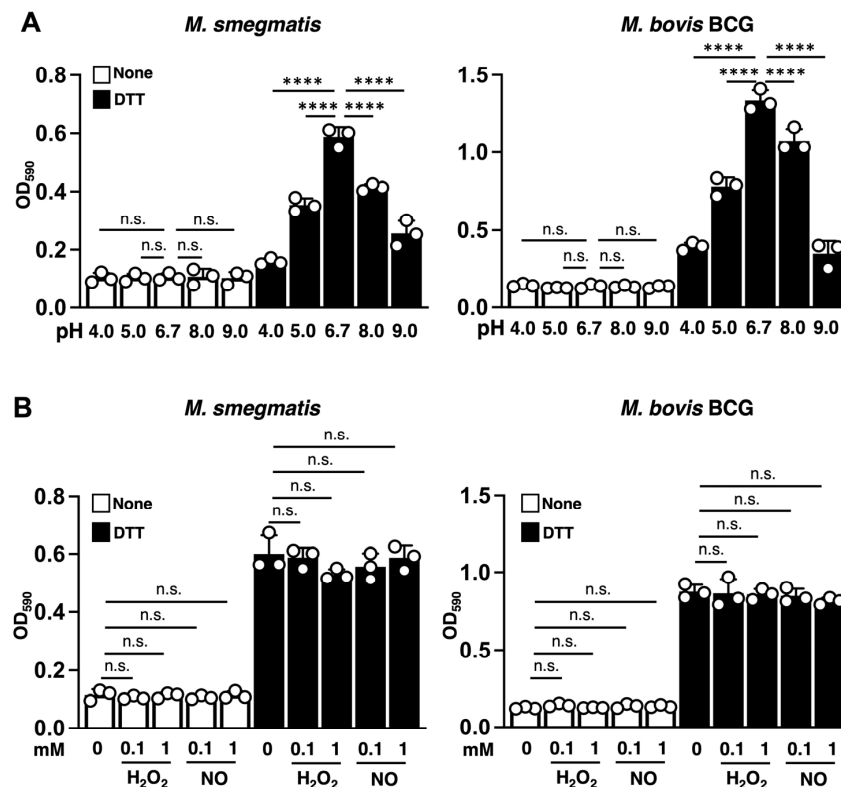


Figure 3. Analysis of the effect of pH and NO stimuli on the formation of mycobacterial biofilms. (A) Effect of pH on mycobacterial biofilm formation. Msm and BCG cells were stimulated with or without DTT (10 mM) at pH 4, 5, 6.7, 8, and 9. (B) Effects of oxidative stress on mycobacterial biofilm formation. Msm and BCG were stimulated with or without DTT (10 mM) in the presence of H₂O₂ and NO (0.1 and 1 mM, respectively). All experiments were performed in triplicate and each experiment was performed at least twice. Statistical analysis was performed using one-way ANOVA test and Tukey's test for multiple comparisons: **** $p < 0.0001$. n.s., not significant.

3.2. Biofilms Inhibit Capture of Mycobacteria by Macrophages

Bacterial biofilms are thought to act as a barrier against host defenses; thus, we hypothesized that mycobacterial biofilms physically protect bacilli from contact with phagocytes. Scanning electron microscopy (SEM) observations revealed that during biofilm induction with BCG, extracellular components surrounded the bacterial cells in some areas but did not completely cover them (Figure S2). We then investigated whether mycobacteria escaped macrophage capture under biofilm-forming conditions. To quantitatively assess the capture ability of the macrophages, we constructed a chromosomally integrating plasmid encoding GFP. Msm and BCG were transformed with the plasmid, resulting in constitutive GFP expression driven by the *smyc* promoter (designated as Msm/*Psmyc*:GFP and BCG/*Psmyc*:GFP, respectively). Fluorescence-assisted cell sorting (FACS) analysis showed that the levels of GFP fluorescence intensity in the transformed mycobacteria were higher than those in the control strain (Figure 4A). We then attempted to establish an evaluation system for the capture of mycobacteria by macrophages. Briefly, macrophages were co-cultured with GFP-expressing mycobacteria. FACS detected GFP-positive cells among viable macrophages (propidium iodide (PI)-negative cells), showing that the capture of mycobacteria by macrophages could be assessed by FACS analysis (Figure 4B). Subsequently, we measured the percentage of GFP-positive macrophages in the presence and absence of biofilms. The results showed that the presence of biofilms significantly reduced the rate of macrophage capture (Figure 4C and Table 1). Together with SEM observations of the reductive stress-induced extracellular components surrounding the mycobacteria under

biofilm-forming conditions (Figure S2), our findings collectively suggest that the biofilm matrix functions as a physical barrier that limits the access of macrophages to mycobacteria.

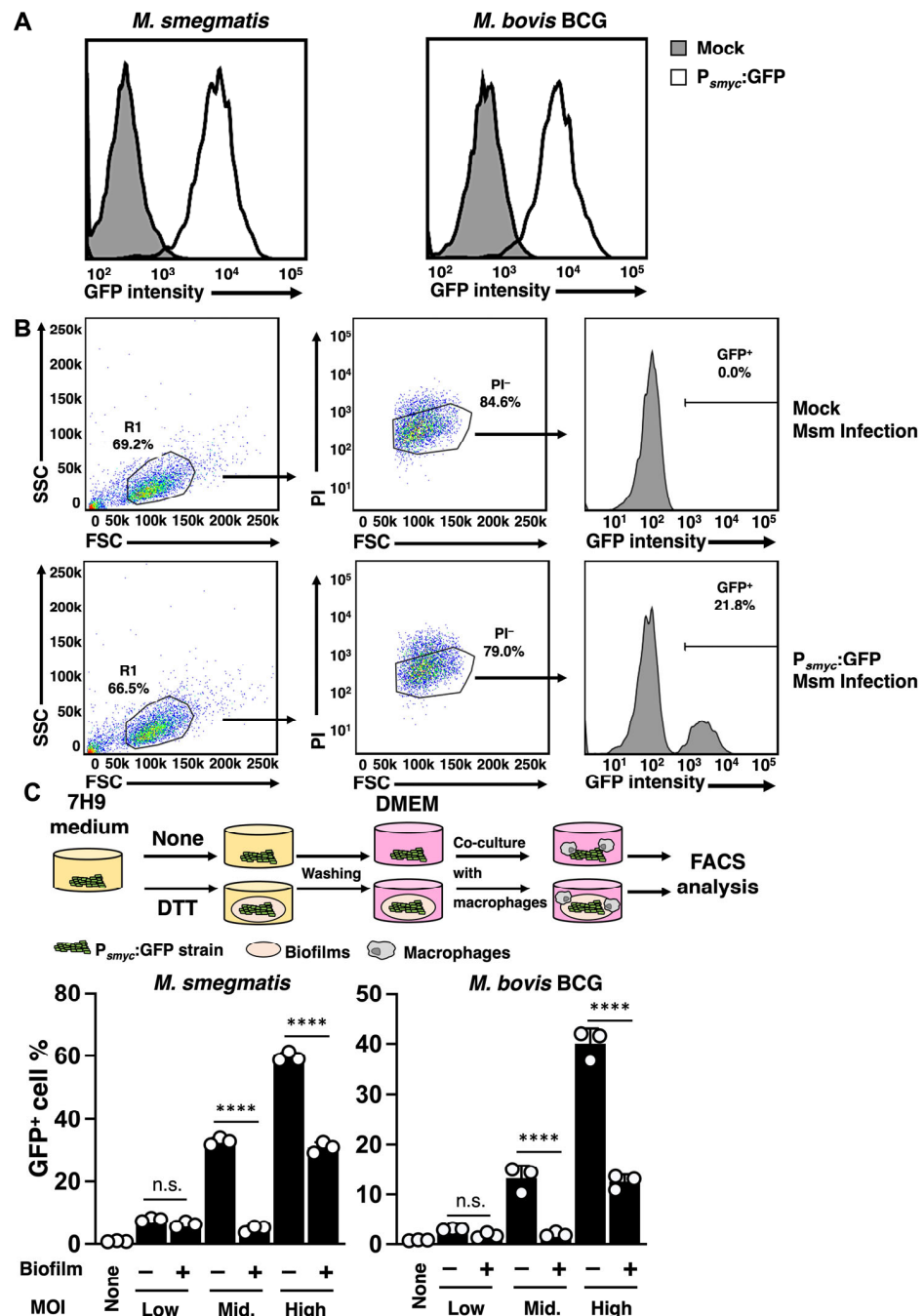


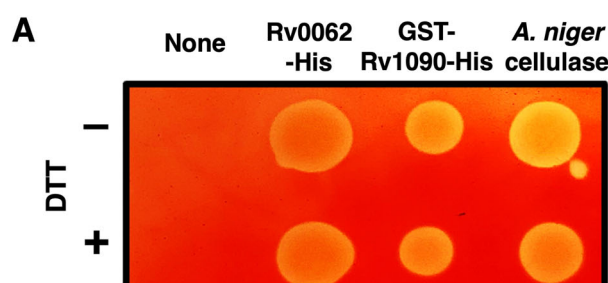
Figure 4. Mycobacterial biofilms prevent capture of bacteria by myeloid cells. (A) Analysis of GFP expression in wild-type and Msm/ P_{smyc} :GFP or BCG/ P_{smyc} :GFP using FACS. (B) Analysis gates for the calculation of GFP-positive RAW264.7 macrophages after co-culture with mock or GFP-labeled Msm for 4 h using FACS. (C) Experimental scheme for determining the ratio of GFP-positive cells after co-culture of RAW264.7 macrophages with several doses of Msm/ P_{smyc} :GFP and BCG/ P_{smyc} :GFP with or without 10 mM DTT at low (multiplicity of infection [MOI] $\approx$ 1), intermediate (MOI $\approx$ 3), and high (MOI $\approx$ 10) doses. The y-axis indicates the percentage of GFP-positive (bacteria positive) events among viable (PI-) RAW264.7 cells after gating as shown in panel (B). All experiments were performed in triplicate, and each experiment was performed at least twice. Statistical analysis was performed using one-way ANOVA and Tukey's test for multiple comparisons: **** $p < 0.0001$. n.s., not significant.

Table 1. Bacterial concentrations (CFU $\times 10^5$ /well) of Msm and BCG.

Species	Condition	Low	Middle	High
Msm	Planktonic	1.2 ± 0.2	3.4 ± 1.1	9.3 ± 3.2
	Biofilm	0.9 ± 0.1	2.9 ± 0.8	9.9 ± 2.3
BCG	Planktonic	0.9 ± 0.2	2.8 ± 1.3	8.9 ± 2.5
	Biofilm	1.1 ± 0.3	2.5 ± 1.1	9.0 ± 3.1

3.3. Cellulase Impairs the Protective Functions of Mycobacterial Biofilms

We found that mycobacterial biofilms were susceptible to cellulase (Figure S1C), which effectively inhibited biofilm formation. To demonstrate the role of reductive stress-induced biofilms in protecting mycobacteria from host defenses, we tested whether biofilm removal by cellulases promoted macrophage capture. We compared the ability of various cellulases to inhibit mycobacterial biofilm formation. Commercially available fungal cellulases derived from *T. viride*, *T. reesei*, and *A. niger* were tested in addition to the in-house mycobacterial cellulases Rv0062 and Rv1090 derived from Mtb. The mycobacterial cellulases were produced in an *Escherichia coli* Rosetta2 with the pET49B plasmid encoding Rv1090 and Rv0062 (Figure S3A). Rv1090 was fused with GST to improve its solubility because intact Rv1090 aggregates in the insoluble fraction after the lysis of *E. coli* cells. The cellulase activities of Rv0062 and Rv1090 were then examined using a CMC agar assay combined with Congo red, which binds to cellulose. In this assay, if the purified proteins spotted on the CMC agar possess cellulase activity, only those spots will not be stained with Congo red. The results showed that Rv0062 and Rv1090 exhibited cellulase activities comparable to those of fungal cellulases in the presence or absence of DTT (Figure 5A). Next, we tested the ability of cellulases to inhibit biofilm formation. Cellulases derived from *T. viride* and *A. niger* strongly degraded biofilm (Figure 5B). In contrast, cellulases from Mtb and *T. reesei* were unable to inhibit biofilm formation in the same concentration range. Moreover, mycobacterial cellulase Rv0062 inhibited biofilm formation at high concentrations, whereas Rv1090 did not (Figure S3B). We also confirmed that the cellulase derived from *A. niger* did not prevent mycobacterial growth (Figure S3C). As shown in Figure 4, biofilms protect bacilli from macrophage capture; thus, we investigated whether cellulase treatment impairs biofilm-mediated protection, resulting in enhanced macrophage capture. In the capture assay using Msm/*P_{smyc}*:GFP or BCG/*P_{smyc}*:GFP, *A. niger* cellulase increased the capture of bacilli by macrophages, suggesting that biofilm removal improved macrophage-mediated bacilli capture (Figure 5C). These results suggest that mycobacterial biofilms protect bacilli from being captured by macrophages.

**Figure 5.** Cont.

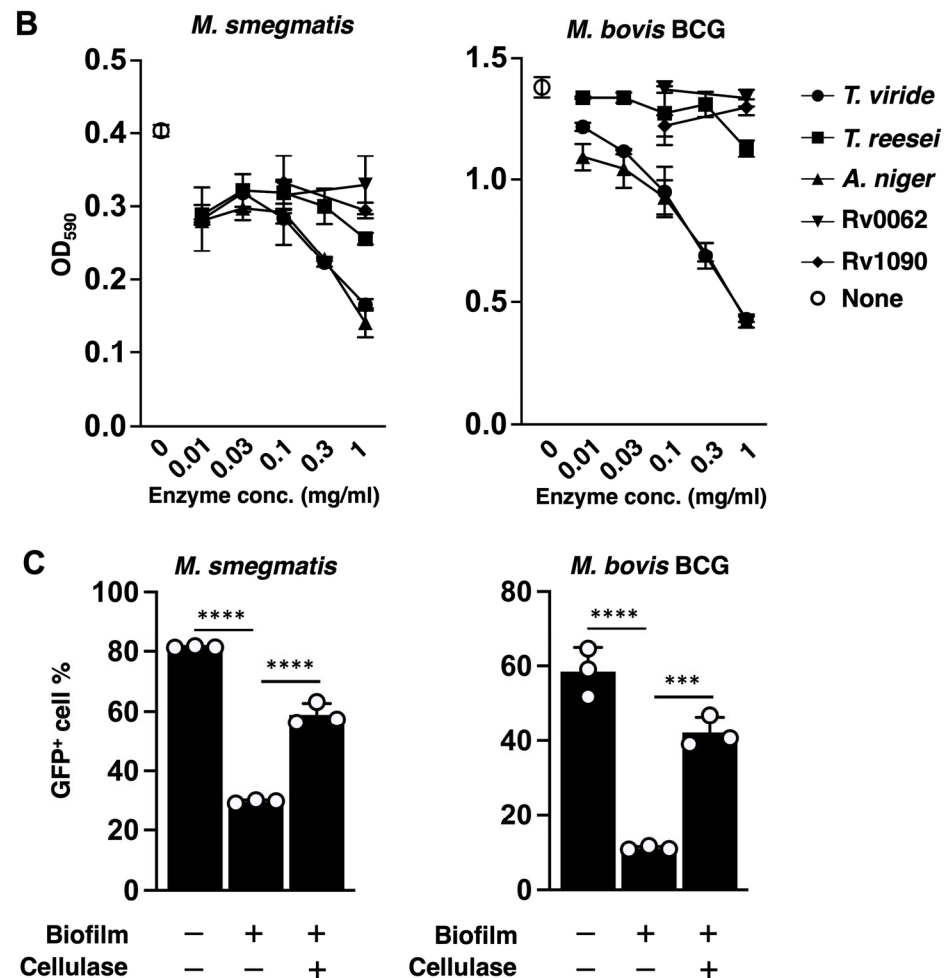


Figure 5. Fungal and mycobacterial cellulases inhibit biofilm formation. (A) Evaluation of the cellulase activity of Rv0062-His-tag, GST-tag-Rv1090-His-tag, and fungal cellulase (5 μ L per spot, 10 mg/mL enzyme concentration) using CMC assay with or without 10 mM DTT. (B) Comparative analysis of cellulase activity. Crystal violet staining assay of Msm and BCG cells exposed to 10 mM DTT with or without cellulase (0.01, 0.03, 0.1, 0.3, or 1 mg/mL). (C) Ratio of GFP-positive cells following co-culture of RAW264.7 macrophages with several doses of Msm/*P_{smyc}*:GFP or BCG/*P_{smyc}*:GFP in the presence or absence of DTT (10 mM) and/or cellulase from *Aspergillus niger*. Experiments were performed in duplicate (B) and in triplicate (C) and each experiment was performed at least twice. Statistical analysis was performed using one-way ANOVA and Tukey's test for multiple comparisons: *** $p < 0.001$, **** $p < 0.0001$.

3.4. Biofilms Protect Mycobacteria from NO Damage, Yet Do Not Prevent Damage Due to Exposure to Acidic pH

Mycobacteria are damaged by exposure to acidic pH and NO in vivo. To evaluate whether biofilms protect mycobacteria from these lethal factors, we constructed a system to assess the levels of acidic pH and NO stress to which mycobacteria were exposed, as indicated by GFP expression. We established Msm and BCG strains carrying the *GFP* gene under the control of the *aprA* promoter, which expressed GFP under acidic pH stress. This cassette was designated as *P_{aprA}*:GFP, and the mycobacteria carrying it were described as Msm/*P_{aprA}*:GFP and BCG/*P_{aprA}*:GFP. Under acidic pH conditions, the GFP expression levels of the strains carrying *P_{aprA}*:GFP significantly increased (Figure S4A). We then evaluated the protective function of mycobacterial biofilms against acidic pH by measuring the GFP fluorescence intensity. The results showed that GFP expression increased in Msm/*P_{aprA}*:GFP and BCG/*P_{aprA}*:GFP cells regardless of biofilm formation under acidic

pH conditions (Figure S4B, left panel). There was no significant difference in the ratio of the fluorescence intensity between the presence and absence of biofilms (Figure S4B, right panel). Similarly, the survival of mycobacteria within biofilms was comparable to that of planktonic bacteria under acidic pH conditions (Figure S4C). We then established mycobacteria expressing the *GFP* gene under the control of the *icl1* promoter (referred to as Msm/*P_{icl1}*:GFP and BCG/*P_{icl1}*:GFP) and monitored the levels of NO to which the mycobacteria were exposed by measuring the GFP expression levels (Figure 6A). In the absence of biofilms, we observed an increase in the GFP expression in Msm/*P_{icl1}*:GFP and BCG/*P_{icl1}*:GFP under NO exposure, whereas an increase in GFP expression of mycobacteria was not detected in the presence of biofilms (Figure 6B, left panel). Moreover, the ratio of the increase in fluorescence intensity of each mycobacterium under NO stress was significantly suppressed by biofilm formation (Figure 6B, right panel). Consistent with these findings, the survival rate of mycobacteria within the biofilm under NO stress was higher than that of planktonic mycobacteria (Figure 6C). These data indicate that mycobacterial biofilms improve mycobacterial survival by protecting them from NO exposure, although they cannot prevent acidic pH stress. Moreover, cellulase treatments increased NO-inducible GFP expression of Msm/*P_{icl1}*:GFP and BCG/*P_{icl1}*:GFP under biofilm formation. These results indicate that biofilm removal enhanced the exposure of mycobacteria to NO (Figure 6D), supporting the conclusion that mycobacterial biofilms protect bacteria from NO exposure.

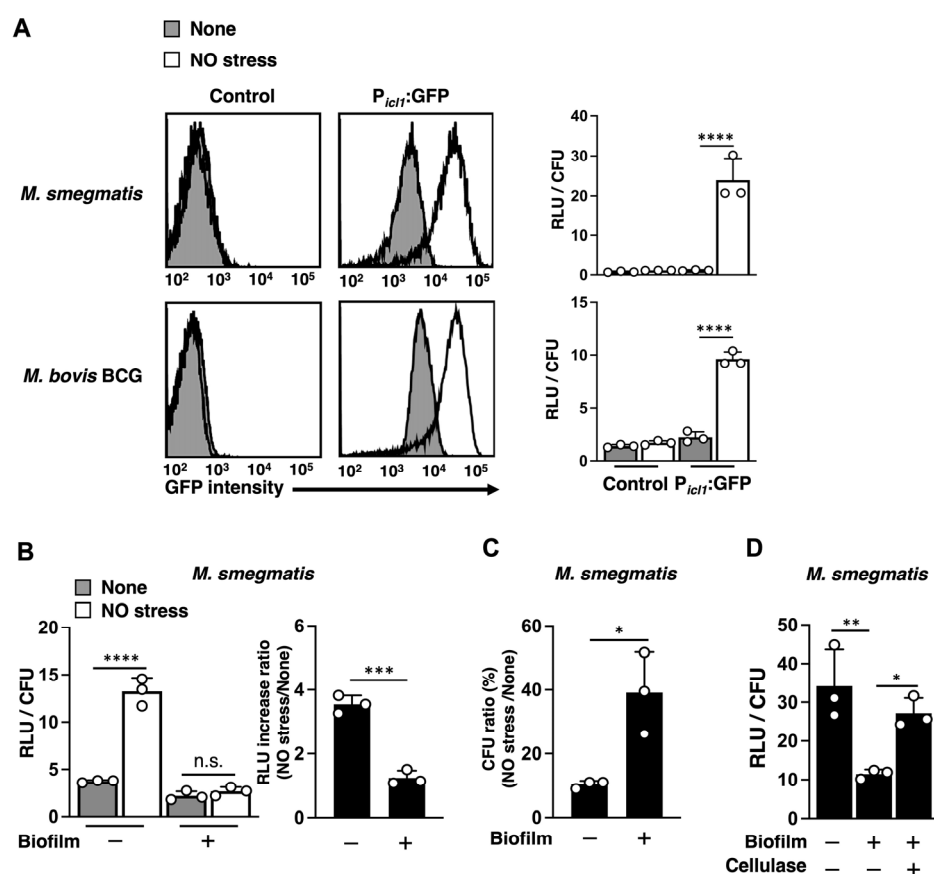


Figure 6. Cont.

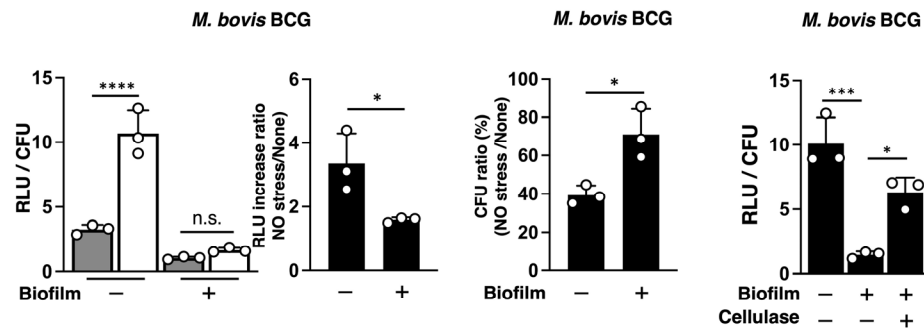


Figure 6. Mycobacterial biofilms prevent mycobacteria from exposure to NO. **(A)** Evaluation of GFP expression in wild-type strains and Msm/*P_{icl1}*:GFP or BCG/*P_{icl1}*:GFP in the presence or absence of NO stress using fluorescence-assisted cell sorting. **(B)** Measurement of GFP expression in wild-type strains and Msm/*P_{icl1}*:GFP or BCG/*P_{icl1}*:GFP, with or without DTT, and in the presence or absence of NO stress, using a NIVO™ plate reader (left panel). The GFP expression ratio of wild-type strains and Msm/*P_{icl1}*:GFP or BCG/*P_{icl1}*:GFP with or without DTT (10 mM) in the presence or absence of NO stress (right panel). **(C)** Survival ratio of Msm/*P_{icl1}*:GFP or BCG/*P_{icl1}*:GFP cells treated with or without DTT under the absence of NO stress. **(D)** GFP expression ratios of Msm/*P_{icl1}*:GFP or BCG/*P_{icl1}*:GFP with or without treatment with DTT/cellulase under NO stress. All experiments were performed in triplicate and each experiment was performed at least twice. Statistical analysis was performed using one-way ANOVA and Tukey's test for multiple comparisons: **** $p < 0.0001$. n.s., not significant. When statistical analysis was performed using Student's *t*-test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. n.s., not significant.

4. Discussion

Mycobacteria form cellulose-containing biofilms in response to reductive stimuli. Biofilms can also be formed within infected organs, conferring resistance to therapeutic agents. However, their functions in host defense reactions have not been analyzed. In this study, we constructed constitutive or NO-inducible GFP-expressing mycobacteria and showed that reductive stress-induced mycobacterial biofilms protected mycobacteria from macrophage capture and NO exposure. Although DTT has been reported to induce mycobacterial biofilm formation as a reductive stimulus, we found that exogenous GSH also promoted biofilm formation under our experimental conditions. However, it remains unclear where mycobacteria are exposed to reductive stimuli in vivo. While it has been reported that mycobacteria can form extracellular biofilms on airway epithelial cells [30], there are also reports that *Pseudomonas*, *Streptococcus*, and *Salmonella* spp. form biofilm-like structures intracellularly [31,32], suggesting that Mtb may have the potential to form biofilms both intracellularly and extracellularly. Thus, clarifying the mechanisms of in vivo biofilm formation remains crucial for uncovering mycobacterial survival strategies and the processes underlying pathogenesis within the host.

To understand the role of mycobacterial biofilms in vivo, it is essential to investigate the mechanisms underlying biofilm formation and degradation. During the bacterial biofilm life cycle, partial degradation of the biofilm matrix is required for bacterial dispersal and transmission [33]. Mtb possesses multiple cellulase-like genes, such as Rv0062 and Rv1090, but their in vivo functions remain unclear. Previous studies have reported that Mtb strains overexpressing the secreted forms of Rv0062 or Rv1090 exhibit reduced virulence in a mouse infection model [11], suggesting that these enzymes may attenuate virulence by degrading biofilms and disrupting their bacterial protective structure. However, this finding alone does not support the conclusion that cellulases simply weaken bacterial defenses, because biofilm degradation is also a necessary process for bacterial dissemination and the spread of infection. Therefore, it is important to determine whether mycobacterial cellulases facilitate dissemination. This question can be addressed by examining their

in vivo expression and testing whether cellulase-deficient mutants exhibit reduced dissemination/spread compared with the wild-type strain. Functional analysis of these cellulases in Mtb-infected lungs may provide important insights into the mechanisms by which Mtb disseminates within the host and contributes to inter-individual transmission. Rv0062 is conserved among non-pathogenic mycobacteria, such as Msm and BCG [11,34], suggesting that these species also possess the ability to degrade cellulose. In contrast, Rv1090 is not conserved in these species and is likely an evolutionarily acquired Mtb-specific gene. Thus, Rv0062 may function as a biofilm-remodeling enzyme conserved across the genus *Mycobacterium*, whereas Rv1090 may have evolved a distinct role associated with Mtb pathogenicity and persistence.

We demonstrate that mycobacterial biofilms confer protection against NO stress. NO is a highly reactive molecule that exerts bactericidal effects by interacting with nucleic acids and proteins. Although the target molecules of NO and the components of mycobacterial biofilms have not been fully elucidated, albumin, a key factor in biofilm formation, is present within the biofilm matrix [16]. Previous reports have demonstrated that albumin has a high affinity for NO [35]. Thus, we speculated that albumin may function as a decoy against NO stress in mycobacteria. However, our data showed that mycobacterial biofilms did not completely protect the bacilli from NO-mediated killing. This incomplete protection may reflect the presence of pores and channels in the biofilm matrix. Pores and channels exist in bacterial biofilms and serve as conduits for the transport of nutrients, waste products, and signaling molecules [36], suggesting the possibility that these pores may permit the diffusion of NO into the biofilm interior.

Given the complex nature of the in vivo environment, we analyzed the effects of a combination of these stimuli on biofilm formation. Similarly, drastic environmental changes such as immune responses and inflammation occur in infected organs. Phagocytes produce oxidative factors that kill bacteria, and several bacteria induce biofilm formation to survive in response to this stress [14]. However, oxidative stress was not required for mycobacterial biofilm formation (Figure 3B) [15]. Cytokines and hormones have also been reported to promote periodontal biofilm formation [37], and the effects of these biological factors on mycobacterial biofilm formation should be further investigated. Quorum sensing has been reported to contribute to biofilm formation in several bacteria such as *Vibrio* and *Pseudomonas* spp. [38]. Planktonic mycobacteria can form biofilms upon the addition of the supernatant from the biofilm culture, suggesting that quorum sensing may be associated with the formation of mycobacterial biofilms [39]. It is possible that quorum sensing also plays an important role in biofilm formation in vivo. Therefore, further studies are required to identify the factors involved in quorum sensing and their synthetic pathways.

Cellulases almost completely inhibited the formation of mycobacterial biofilms despite the presence of nucleic acids, proteins, and other components in the biofilm (Figure S1C), indicating that cellulose may be the ground for biofilm formation. To initiate biofilm formation, bacteria are commonly thought to bind to the surface of an object using pili, flagella, or adhesion proteins; mycobacteria do not possess these adhesion factors. Hydrophilic oligosaccharides in the LPS of *E. coli* have been reported to contribute to bacterial adhesion to material surfaces [40]. Mycobacteria produce polysaccharides, including cellulose, on the exterior of their cell walls. Cellulose promotes the adherence of *Salmonella* spp. to the surface of an object [41], suggesting that cellulose in mycobacterial biofilms may also act as a base for biofilm formation.

Due to the logistical constraints of BSL-3 environments, the downstream functional assays in this study were limited to attenuated strains and RAW264.7 cells. Although reductive stress-induced biofilm formation was confirmed in virulent *M. tuberculosis*, the distinct interactions between virulent strains and human macrophages remain to be fully

elucidated. Further study under clinically relevant conditions will be essential to generalize these findings to human tuberculosis infection.

In conclusion, our results demonstrate that mycobacterial biofilms act as a barrier against host defenses and help mycobacteria survive in hostile environments. Therefore, biofilm removal may be a novel therapeutic strategy for mycobacterial infections by enhancing host immunity.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/applmicrobiol6030035/s1>, Figure S1: Characteristics of mycobacterial biofilms are conserved across species, Figure S2: Ultrastructure of the biofilm produced by BCG, Figure S3: High concentrations of mycobacterial cellulase can detach biofilms, Figure S4: Mycobacterial biofilms do not protect mycobacteria from acidic pH stress. Table S1: Bacterial strains and plasmids used in this study, Table S2: Primers used in this study.

Author Contributions: S.T., Conceptualization, Experiment design, Investigation, Data curation, Formal analysis, Writing—original draft and review and editing; K.Y., Methodology, Supervision, Writing—original draft and review and editing; M.A., Supervision, Writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: All data generated or analyzed during this study are included in this published article.

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Conflicts of Interest: The authors declare no conflicts of interest.

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