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Letter

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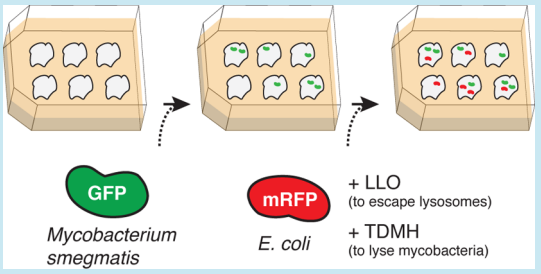
In Situ Characterization of Mycobacterial Growth Inhibition by Lytic Enzymes Expressed in Vectorized *E. coli*

Iva Atanaskovic,[†,§] Amel Camélia Bencherif,[†,§] Matthew Deyell,[†] Sebastián Jaramillo-Riveri,[†] Marguerite Benony,[†] Aude G. Bernheim,[†] Vincent K. Libis,[†] Nicolas Koutsoubelis,[†] Yonatan Zegman,[†] Anne C. Löchner,[†] Clovis Basier,[†] Idonnya Aghoghogbe,[†] Zoran S. Marinkovic,[†] Sarah Zahra,[†] Matthias Toulouze,[†] Ariel B. Lindner,*[,†,‡] and Edwin H. Wintermute*[,†,‡]

[†]2013 Paris Bettencourt iGEM team, Centre for Research and Interdisciplinarity, Paris Descartes University, 75014 Paris, France

[‡]U1001 Institut National de la Santé et de la Recherche Médicale, 75014 Paris, France

ABSTRACT: The emergence of extremely drug resistant *Mycobacterium tuberculosis* necessitates new strategies to combat the pathogen. Engineered bacteria may serve as vectors to deliver proteins to human cells, including mycobacteria-infected macrophages. In this work, we target *Mycobacterium smegmatis*, a nonpathogenic tuberculosis model, with *E. coli* modified to express trehalose dimycolate hydrolase (TDMH), a membrane-lysing serine esterase. We show that TDMH-expressing *E. coli* are capable of lysing mycobacteria *in vitro* and at low pH. Vectorized *E. coli* producing TDMH were found suppress the proliferation of mycobacteria in infected macrophages.



KEYWORDS: *mycobacteria, TDMH, LLO, protein vectors, macrophages, tuberculosis*

M. tuberculosis infects macrophages within the lungs where it is difficult to reach with conventional antibiotics. This problem is compounded by the highly impermeable mycobacterial membrane, which also resists the normal process of phagocytosis. The recently discovered enzyme TDMH, a serine esterase, can enzymatically lyse the mycobacterial cell envelope by digesting outer-membrane trehalose mycolates.[1]

Previous work has shown that engineered *E. coli* can serve as a vector to deliver active proteins to the macrophage cytosol.[2] Briefly, lysteriolysin O (LLO) forms large pores in the phagosomal membrane that allow the passage of proteins to the cytosol. *E. coli* that express LLO are phagocytosed and lysed, causing the release of both LLO porin and their protein payload.

In this work, we explore the feasibility of using *E. coli* as a vector to deliver TDMH to mycobacteria-infected macrophages. We show that individual *E. coli* are capable of expressing sufficient TDMH to lyse more than 10 mycobacteria in mixed cultures and that TDMH is active at low pH matching the phagosomal environment. Further, we show that engineered *E. coli* can colocalize with mycobacteria and can halt mycobacterial spread in cultured macrophages.

RESULTS AND DISCUSSION

Following Yang[1] and Higgins,[2] we created an *E. coli* strain capable of inducible expression of both TDMH and LLO. *M. smegmatis* served as a nonvirulent model for *M. tuberculosis* because it is known to exhibit comparable membrane structure and macrophage infection dynamics.[3]

We first sought to verify that TDMH-expressing *E. coli* were able to kill mycobacteria in coculture (Figure 1). We achieved nearly 99% killing of mycobacteria after 6 h when *E. coli* and

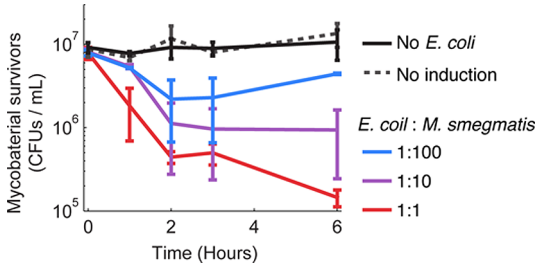


Figure 1. TDMH-expressing *E. coli* kill mycobacteria in mixed cultures. *M. smegmatis* and *E. coli* populations were mixed in LB at defined ratios and TDMH expression was induced with IPTG. Viability was assayed at defined time points by serial dilution and plating. Nalidixic acid plates were used to select and count only *M. smegmatis* from the mixed population. Nearly 99% killing of *M. smegmatis* was recorded after 6 h when the strains were mixed in equal proportion (red line). Significant killing was also apparent for *E. coli: M. smegmatis* ratios of 1:10 (purple line) and 1:100 (blue line). No change in viability was recorded when *M. smegmatis* were cultured alone (black line) or mixed 1:1 with uninduced *E. coli* (gray dotted line). Error bars represent 95% confidence intervals calculated from at least 3 biological replicates.

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mycobacteria were mixed in equal proportion and TDMH expression was induced. Uninduced *E. coli* produced no significant killing. Varying the ratio of *E. coli* to *M. smegmatis* indicated that effective killing required approximately one fully induced *E. coli* for every 10 mycobacteria.

The phagosomal pH is significantly more acidic than batch culture media, varying from 4.8 to 5.4 under most conditions.[4] We next tested if TDMH-mediated killing could function at low pH (Figure 2). *M. smegmatis* growth was measured with and

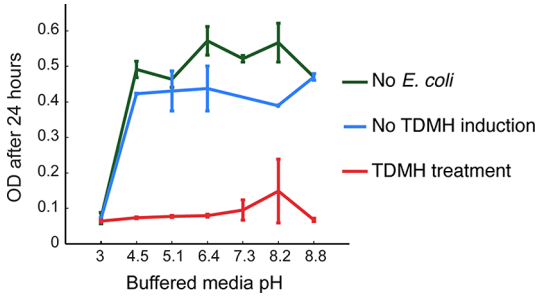

Figure 2. TDMH functions to inhibit mycobacterial growth at low pH. Media supernatant from induced and uninduced TDMH-producing *E. coli* was applied to *M. smegmatis* cultures growing in LB buffered to the indicated pH. Mycobacterial growth was assayed by a plate reader after 24 h. At pH 3, no growth was detected for any treatment. Mycobacteria treated with TDMH-induced *E. coli* supernatant (red line) showed no growth after 24 h at any pH tested. Mycobacteria treated with only LB (green line) or supernatant from uninduced *E. coli* (blue line) grew to saturation. The effect of TDMH on mycobacterial growth was independent of pH.

without supernatant collected from TDMH-expressing *E. coli* in media buffered to pH values ranging from 3 to 8.8. At very low pH values, *M. smegmatis* was unable to grow regardless of TDMH treatment. At pH levels from 4.5−8.8, *M. smegmatis* was able to grow and TDMH was functional as a growth inhibitor. This suggests that TDMH activity is retained at phagosomal pH.

We next evaluated the capacity of vectorized *E. coli* to colocalize with mycobacteria within infected macrophages (Figure 3a, b). We exposed a population of macrophages first to GFP-expressing *M. smegmatis*, then to RFP-expressing *E. coli*. Using fluorescence microscopy, we visually scored the bacteria taken up by each macrophage. We found that 26% of macrophages contained both *E. coli* and *M. smegmatis* while only 4% contained *M. smegmatis* alone (n = 12 807). The high rate of coinfection indicates that macrophages containing *M. smegmatis* remain active and are able to take up vectorized *E. coli* in most cases.

Finally, we applied *E. coli* expressing both TDMH and LLO to treat macrophages preinfected with mycobacteria (Figure 3c). Addition of vectorized *E. coli* significantly reduced mycobacterial infection rates in a population of macrophages after 3 h, as determined by fluorescence microscopy. A control population of macrophages, infected with only GFP-expressing *M. smegmatis*, showed a significant increase in mycobacterial infection rate over the same time period. This suggests that vectorized *E. coli* are able to slow or reverse the spread of mycobacteria within macrophages.

The antimycobacterial activity of vectorized *E. coli* might be improved by optimizing the activity, expression and mechanism of the relevant enzymes. A variety of bacteriolytic enzymes are in common laboratory use and could be adapted for this

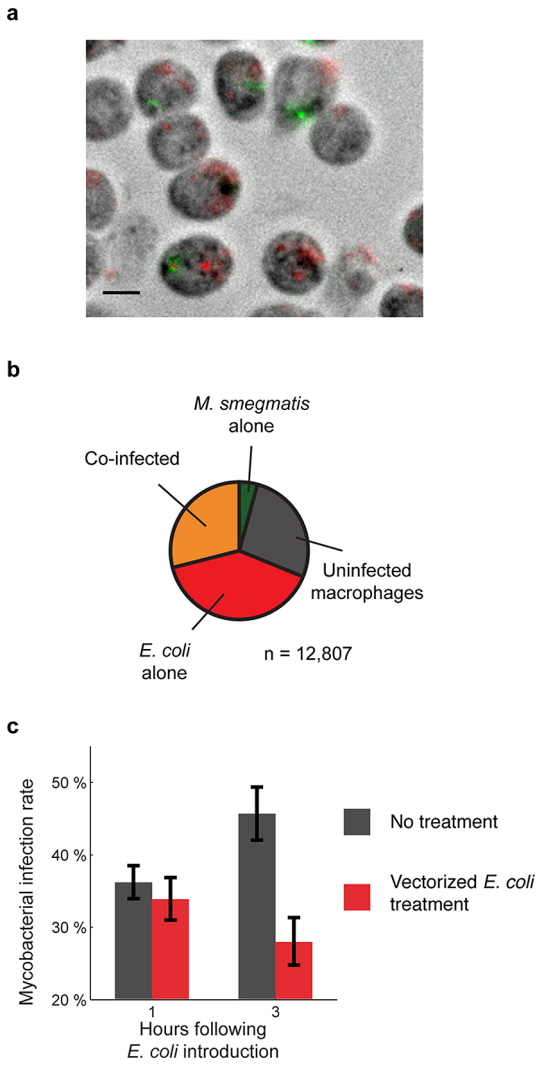

Figure 3. Vectorized *E. coli* localize to mycobacteria-infected macrophages and slow the spread of mycobacteria in a macrophage population. (a) *E. coli* expressing LLO and TDMH (labeled with RFP) were introduced to macrophages along with *M. smegmatis* (labeled with GFP). After 1 h, colocalization was scored by fluorescence microscopy at 40× magnification. Scale bar is 5 μM. (b) 26% of imaged macrophages contained *E. coli* and *M. smegmatis*, compared to 4% containing *M. smegmatis* alone. This indicates that *E. coli* can enter mycobacteria-infected macrophages at high frequency. (c) Mycobacterial localization in macrophages was scored 1 h following coinfection with *E. coli* and again after 3 h. Mycobacterial infection rates declined in a macrophage population treated with vectorized *E. coli* (red bars). In control macrophages treated with mycobacteria alone, mycobacterial infection rated increases significantly after 3 h (gray bars). This may indicate the activity of TDMH in lysing mycobacteria and inhibiting growth. Error bars represent 95% confidence intervals of at least 3 biological replicates.

purpose. Cell-envelope degrading enzymes like TDMH may be particularly valuable if they can act synergistically with existing antimycobacterial drugs like isoniazid or ethambutol that target cell-envelope biogenesis. Potential synergy may also be found with the oxidative, acidic, and enzymatic stresses that weaken bacterial membranes within the phagosome.

While synthetic *E. coli* have previously been proposed as antimicrobials, a common challenge is directing their activity in a complex microenvironment.[5] In the case of intraphagosomal mycobacteria, we have shown that it is possible to make use of

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the existing immune response to target a bacterial payload. Vectorized *E. coli*, by delivering functional protein systems to the intracellular macrophage, can enhance its natural microbicidal activity.

METHODS

Strains and Fluorescent Labeling. The BL21(DE3) strain of *E. coli* was used for all experiments. RFP labeling of *E. coli* was achieved with a pCOLA plasmid carrying BBa_J23102 from the Registry of Standard Biological Parts. *Mycobacterium smegmatis* MC2 155 labeled with pMyco-*gfp*[6] was a gift from Stéphane Canaan (CNRS). J774 macrophages were obtained from Nicolas Hegerle and Nicole Guiso (Institut Pasteur).

TDMH and LLO Expression. TDMH expression was achieved with the IPTG-inducible plasmid pET21b carrying Msmeg_1529 (TDMH), provided by Dr. Anil Ojha (University of Pittsburgh). Constitutive LLO expression was achieved with the plasmid pACYC184 encoding *hly* (LLO), provided by Dr. Daniel A Portnoy (UC Berkeley).

Culture Conditions and Killing Assay. *M. smegmatis* and *E. coli* were grown separately in LB media to saturation in biological triplicates. The strains were diluted by a factor of 100 and mixed 1:1 in a 10 mL volume, and full induction of TDMH expression was achieved with 1 mM IPTG. At defined time periods, viable *E. coli* and *M. smegmatis* were assayed by serial dilution and selective plating. Nalidixic acid (3 μg/mL) was used to select and count only *M. smegmatis* colony-forming units in the mixed cultures. Kanamycin (100 μg/mL) similarly selected for only *E. coli* carrying pCOLA-RFP.

pH Dependence of TDMH Activity. *M. smegmatis* was resuspended at an initial OD of 0.05 in LB buffered with phosphate to a range of pH values. Filter-sterilized supernatant was collected from saturated *E. coli* cultures induced for TDMH expression, or uninduced cultures as a control. Supernatant (20 μL) was added to 200 μL of *M. smegmatis* culture and growth was followed by OD on a plate reader after 24 h. In all cases, measurements were performed in biological triplicate.

Macrophage Experiments and Microscopy. Macrophages were grown in 24 well plates in RPMI 1640 media supplemented with 1% glutamine, 10% of fetal bovine serum, 1% HEPES, and 1% sodium pyruvate. Experiments began when the cells approached 90% confluence.

E. coli and *M. smegmatis* were grown to saturation, washed 3 times with RPMI media, and resuspended at an OD of 0.1. 100 μL of each bacterial strain was added to 1 mL of macrophage culture. Cell mixtures were incubated for 1 h without agitation (37 °C, 5% CO2), then washed 10 times with PBS and resuspended in fresh RPMI.

For the coinfection experiments (Figure 3a, b), the presence of *E. coli* or *M. smegmatis* was scored visually using a Nikon Eclipse Ti-E inverted fluorescent microscope. We collected 119 images from 6 separate wells representing 3 biological replicates. In total, 12 807 macrophages were scored.

For the *in situ* growth-inhibition experiments (Figure 3c), GFP-expressing *M. smegmatis* were grown to saturation, washed, and introduced to macrophages as above. Macrophages were either untreated or preinfected with vectorized *E. coli* expressing TDMH, LLO and RFP. The percentage of macrophages infected with *M. smegmatis* was scored visually after 1 and 3 h by fluorescence microscopy. At least 500 cells from at least 6 images were scored for each data point.

AUTHOR INFORMATION

Corresponding Authors

*E-mail: ariel.lindner@inserm.fr.

*E-mail: ehwintermute@gmail.com.

Author Contributions

[§]I.A. and C.B. contributed equally to this work. C.B., I.A., and E.H.W. designed the experiments. C.B., I.A., and M.D. conducted the experiments. All authors contributed to the analysis and interpretation of results. I.A., C.B., and E.H.W. prepared the manuscript.

Notes

The authors declare no competing financial interest.

Further discussion of this project and its context within the larger work of the 2013 Paris Bettencourt iGEM team is available via our team Web site at http://2013.igem.org/Team:Paris_Bettencourt.

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