1 **The transcriptional response of *Yersinia pseudotuberculosis* to macrophage-released**

2 **chemicals during growth within synthetic microcolonies**

Running title: Spatially-controlled transcriptional response of *Yersinia*

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Abstract

Yersinia pseudotuberculosis (Yptb) replicates in immune cell-encompassed microcolonies within tissues. Bacterial replication is controlled by protection against neutrophil attack and by macrophage-released antimicrobial factors, such as nitric oxide (NO). During these attacks, bacteria located on the microcolony periphery encounter extracellular signals that differ from those in the interior. To dissect individual microbial populations, γ interferon-activated macrophages were used to challenge microdroplet-grown *Yptb* harboring an NO-responsive mCherry reporter. Subsequently, bacterial subpopulations that hyperactivated the reporter were isolated from droplets composed of a reversible polymer matrix. RNA-seq analysis indicated that induction of nitrosative stress-associated genes was the primary determinant distinguishing peripheral bacteria from the remaining population. In addition, a secondary stress response that induced prophage-associated genes was detected, which could not be traced to either DNA damage or nitrosative stress responses. Activated macrophages also induced the expression of the *Yptb* itaconate degradation enzyme-encoding transcript throughout the entire colony. To determine if itaconate production by the interferon-activated Irg1 protein played a role in restricting *Yptb*, bacteria harboring an itaconate-responsive reporter and *Yptb* mutants defective for itaconate degradation were analyzed during bacterial colonization of the murine spleen. Only a subset of colonies appeared to be exposed to itaconate, which may explain the very small defects exhibited by mutants unable to degrade the interferon-induced macrophage product. These results indicate that the primary response of bacteria to macrophage-elicited factors is likely associated with protection against NO-derived metabolites.

Introduction

During systemic infection, bacterial pathogens can colonize and replicate in deep tissue sites, causing acute or chronic disease (1-3). The colonization site is often surrounded by innate immune cells, which can develop into either abscesses, granulomas, or microcolonies, the latter of which have been referred to as pyogranulomas (4-6). *Yersinia pseudotuberculosis* (*Yptb*) is one such pathogen that establishes disease by forming microcolonies within deep tissues sites during disease (3, 7). After intestinal inoculation of mice, microorganisms replicate in the intestinal lumen and regional lymph nodes, and disseminate via a poorly characterized route into the liver or spleen (8).

During microcolony formation in these deep tissue sites, *Yptb* establishes clonal extracellular microcolonies that develop into lesions densely populated with innate immune cells (3, 7). It is likely that neutrophils are the first immune cells recruited to the focus of replicating bacteria, directly surrounding the growing microcolony. *Yptb* in the periphery of the microcolony are in direct contact with neutrophils, resulting in the translocation of the bacterial Type III Secretion System (T3SS) effector proteins into target host cells, paralyzing cytoskeletal elements and misregulating components of the innate immune response (9-14). As a result, the pathogen disrupts phagocytosis and the production of reactive oxygen species (ROS) by surrounding neutrophils (15). The paralyzed neutrophils, therefore, are unable to limit Yptb growth, likely leading to the subsequent recruitment of additional innate immune cells, such as macrophages and monocytes. This second wave of recruited immune cells surrounds the sphere of neutrophils, resulting in a complex, multilayered structure.

The recruitment of myeloid cells around a cluster of *Yptb* exposes the bacterial microcolony to distinct microenvironments, which drives the phenotypic specialization of

microbial subpopulations that counter host attack. Peripheral bacteria in direct contact with

neutrophils upregulate their T3SS, amplifying the anti-host cell response and rendering the ring

of neutrophils ineffective in clearing the bacteria (7). The cell-associated *Yptb* also contributes

to a larger peripheral subpopulation of bacteria that upregulate the protein Hmp that detoxifies

nitric oxide (NO) gas produced by surrounding macrophages. Consequently, the majority of the

peripheral cells responding to NO do not come into direct contact with any cells, and few, if any,

of the NO-producing host cells are in direct contact with the microcolony. The bacteria at the

center of the microcolony are protected from NO by the Hmp-expressing peripheral bacteria,

providing an example of tissue-associated microbial social behavior in which one subpopulation

protects the inner core. The ability of bacteria to detoxify NO *via* Hmp is important for virulence,

as microcolonies lacking Hmp eventually disintegrate in tissues (7).

Until recently, analysis of bacterial subpopulations was limited to microscopic analysis of

fixed tissue or tissue culture modeling strategies that rely on microbial challenge of cells

incubated in 2-dimensional culture. Analysis of fixed tissue provides a limited opportunity to

perform genetic analysis, and incubations with mammalian cultured monolayers do not

reproduce the three-dimensional (3D) architecture of bacterial microcolonies surrounded by

immune cells in host tissue sites. To bridge the gap between animal infection models and tissue

culture models, we previously developed an *in vitro* system that uses droplet-based microfluidics

to generate matrix-embedded *Yptb* microcolonies, incorporating primary macrophages that bind

to the outside of the droplet (16). Social behavior in response to macrophages could be

reproduced by an NO generator or by macrophages activated to produce inducible nitric oxide

synthase (iNOS). The significance of this model is that topological analysis of *Yptb*-host cell

interaction is unattainable in murine and tissue culture models of infection. It remains unclear

what the growth dynamics of bacterial subpopulations are in this micro-compartment, or if other

macrophage-secreted factors are being produced by macrophages that target *Yptb.*

In this report, we modified the microdroplet culture strategy to efficiently isolate bacterial

subpopulations *via* flow cytometry and perform transcriptional analysis on the *Yptb*

subpopulations. Similar to our original system, social behavior was reproduced in the presence of

a chemical NO generator or by activated macrophages expressing iNOS. Furthermore, we also

provide evidence that the macrophage metabolite itaconate, synthesized as part of the interferon

response, is released into the bacterial microenvironment, and may contribute to control of *Yptb*

growing extracellularly in tissue sites.

Results

Alginate microdroplets support growth of *Yersinia pseudotuberculosis* microcolonies.

Yersinia pseudotuberculosis (*Yptb*) forms clonal microcolonies in the murine spleen that

replicate extracellularly and are surrounded by innate immune cells (3, 7). This 3-dimensional

(3D) topology can be mimicked in culture in which a matrix consisting of 1% ultra-low melt

agarose containing 25% HyStem®-C Hydrogel encapsulates *Yptb* in 65 µm-diameter droplets

(16). This matrix requires heating to isolate bacteria for sorting populations based on fluorescent

protein expression. Unfortunately, chemicals used to inactivate RNase activity resulted in a loss

of protein fluorescence during the heating step (Supplemental Fig. 1). To address this problem,

bioengineered droplets were prepared with alginate, which enables isolation of bacteria without a

heating step (17-20). Alginate is a polysaccharide consisting of guluronate and mannuronate

chains (21). The carboxylate groups of these residues bind most divalent cations, most

commonly Ca^{2+}, thereby allowing the alginate to form a gel immediately.

125  A strategy was devised to enable controlled gelation of alginate droplets using

126 microfluidics, thereby avoiding device clogging (22). To allow controlled gelation, Ca^{2+}/EDTA

127 chelation followed by EDTA acidification is often employed (23), but this strategy was not

128 effective because EDTA blocked bacterial growth in droplets, as reported previously (24, 25). As

129 an alternative, dispersed $CaCO_3$ nanoparticles were incorporated into the system, followed by

130 acidification after microdroplet formation to solubilize Ca^{2+}. To this end, 25 mM $CaCO_3$

131 nanoparticles were added to a matrix consisting of molten 1% alginate, with RGD-alginate as 5%

132 of the polymer content, to allow multivalent adhesion to mammalian integrin receptors (Fig. 1)

133 (16, 17, 26-30). The mixture was then encapsulated into droplets in oil using a microfluidics chip

134 (16), and the alginate was crosslinked by adding dilute acetic acid in oil (Fig. 1A: step 1-3). After

135 crosslinking (Fig. 1A: step 4), the oil was removed, yielding alginate droplets of approximately

136 65 μm in diameter (~144 pl) (Fig. 1B).

137  *Yptb* microcolonies form as clusters in tissue (3, 16). To confirm *Yptb* grows as a cluster

138 inside alginate droplets, *Yptb* expressing GFP (*P*$_{tet}$*::gfp;* Table 2) was encapsulated in droplets,

139 oil was removed, and the droplets were aerated at 26 °C in broth supplemented with 5 mM $CaCl_2$

140 (Materials and Methods). Microcolonies were established that were indistinguishable from the

141 previously described agarose microcolonies (Fig. 1C) (16). Therefore, a tunable and reversible

142 matrix allows microcolony formation of *Yptb*, mimicking topological constraints observed in

143 tissue.

144 **Spatial regulation of *hmp* expression in tissues can be reproduced in alginate**

145 **microdroplets.**

146  To determine if the spatial regulation of *Phmp* observed in tissue can be reproduced in

147 alginate droplets, the WT strain carrying the *gfp*$^{+}$ and *Phmp::mcherry* reporters was encapsulated

in droplets, grown into microcolonies, and exposed to the NO generator DETA-NONOate for 4

hours (Fig. 2A). WT microcolonies showed peripheral expression of *hmp*, while the constitutive

GFP signal was uniform throughout the microcolonies, as observed in tissues (Fig. 2B).   Using a

previously described script to quantitate spatial regulation of *hmp*, the fluorescence at the

periphery was calculated relative to the point of lowest intensity (PLI) internal to the

microcolony (16). There was a significant increase in the periphery vs. PLI ratio in WT

microcolonies in response to DETA-NONOate after 4 hour exposure, indicating peripheral

expression of P*hmp::mcherry* (Fig. 2C; P <0.0001), demonstrating that spatial regulation of *hmp*

can be reproduced in alginate droplets.

Activated BMDMs drive peripheral expression of *hmp* in droplet microcolonies.

To determine if the microcolonies respond to cellularly produced NO, BMDMs activated

by LPS and IFNγ to express inducible NO synthase (iNOS) were added to droplets containing

pregrown microcolonies, mimicking tissue dynamics (16, 31-33). BMDMs bound efficiently to

alginate droplets containing *Yptb* (Fig. 3A). In response to the primed BMDMs, the *Yptb*

microcolonies exhibited spatial regulation of the *Phmp::mcherry* reporter, similar to that

observed with *Yptb* growing in tissue (Fig. 3B) (16). Expression of the *Phmp::mcherry* reporter

required the addition of LPS and IFNγ to BMDMs, as predicted for driving expression of iNOS

(Fig. 3C).

The amount of NO that is generated in tissues surrounding a microcolony is unknown. To

determine the relative amount of NO produced by BMDMs, WT microcolonies harboring the

Phmp::mcherry reporter were challenged in the presence or absence of DETA-NONOate, and

unprimed or LPS/IFNγ-primed BMDMs. The droplets were then dispersed by the addition of

EDTA and analyzed by FACS, determining the median relative fluorescence intensity of

Phmp::mCherry per bacterium (Materials and Methods). *Yptb* originating from WT

microcolonies incubated with 0 mM DETA-NONOate or unprimed BMDMs, showed low

Phmp::mcherry fluorescence (Fig. 3D). After challenge with LPS/IFNγ-primed BMDMs, there

was a significant increase in *Phmp::mcherry* reporter fluorescence compared to its fluorescence

in *Yptb* treated with 1 mM DETA-NONOate (Fig. 3D; p<0.0001)*.* This result is consistent with

LPS/IFNγ-primed BMDMs collectively generating more NO than 1 mM DETA-NONOate.

Therefore, WT microcolonies exhibit spatial regulation of *hmp* in response to activated BMDMs

as seen in tissue, and the relative expression in response to NO secreted by activated BMDMs is

more than that observed in response to 1 mM DETA-NONOate.

Isolation of *Yptb* subpopulations generated in alginate droplets

Yptb on the periphery of a microcolony detoxify NO produced by macrophages, lowering

NO concentrations below levels necessary to induce *Phmp* in the center of the microcolony. The

growth dynamics of these subpopulations within tissue microcolonies is uncertain, and it is not

known if there are transcriptionally-distinct bacterial subpopulations controlled by tissue signals.

Furthermore, RNA-seq transcriptional analysis of *Yptb* grown in tissue has been limited to whole

population analysis without subdividing the response into spatial subpopulations (34). Isolation

of bacterial subpopulations from tissue is difficult, and extensive tissue processing may cause

significant alterations in the bacterial transcriptome. Therefore, reconstructing spatial regulation

in culture is an attractive approach for identifying transcriptional subpopulations. As droplet

culture reconstructs the peripheral (NO-detoxifying bacteria) and central (non-NO-detoxifying)

bacterial subpopulations that are observed in tissues, a route is provided for analyzing spatial

regulation of subpopulations observed with tissue-resident bacteria.

To identify transcriptional differences between *Yptb* peripheral and central populations,

Yptb subpopulations were fluorescence sorted after growth in alginate droplets. After 7 hours of

growth, the WT *gfp$^+$/Phmp::mcherry* strain was incubated for an additional 4 hours with either 0

or 1 mM DETA-NONOate, as well as with unprimed or LPS/IFNγ-primed BMDMs. The

droplets were then disrupted in the presence of RNA*later™* and sorted (Fig. 4A). Live bacteria

were selected by gating for GFP, and peripheral (high Hmp expression; Hmp-HI) versus central

populations (low Hmp expression; Hmp-LO) were sorted based on *Phmp::mcherry* fluorescence

(Fig. 4B-E). Nearly all WT *Yptb gfp+ bacteria* were Hmp-LO without DETA-NONOate (Fig.

4B). After exposure to unprimed BMDMs, most GFP+ bacteria remained Hmp-LO, as expected

(Fig. 4D: about 0.5% are Hmp-HI). The slight increase in bacteria within the Hmp$^+$ gate after

incubation with unprimed BMDMs compared to 0 mM DETA-NONOate (Fig. 4B, 0.095%

Hmp-HI) may result from RGD-alginate activation of the macrophages (35). Conversely,

incubation with either 1 mM DETA-NONOate or LPS/IFNγ-primed BMDMs caused strong

activation of the *Phmp* promoter in a significant portion of bacteria. To focus on the most highly

NO-activated transcripts for transcriptional analysis, GFP+ events were collected from the top

10% of *Phmp::mcherry* fluorescence after treatment with either 1 mM DETA-NONOate (Fig.

4C) or incubation with macrophages treated with LPS/IFN (Fig. 4E).

Yptb in the high _Phmp::mCherry_ gate are transcriptionally distinct from other _Yptb_

Using the strategy described in Fig. 4, bacterial subpopulations were isolated, and

differential RNAseq analysis comparing the *Phmp::mcherry*-HI gate to the *Phmp::mcherry*-LO

gates was completed for 3 paired biological replicates of droplets that were exposed to either

NONOate or IFN-activated BMDMs (Fig. 5) (36). Identical analysis was also performed for

droplet-grown bacteria in the presence or absence of nonactivated-BMDMs. Among the most

highly upregulated genes in response to macrophage droplet adherence were those associated

with a subset of amino acid biosynthesis pathways, especially tryptophan and arginine (Figs.

5A,B).   A similar starvation set was observed for peripherally localized bacteria exposed to

IFNγ/LPS-activated BMDMs, although in the latter case, arginine starvation was no longer

apparent (Fig. 5C,D; Supplemental Dataset 1). In contrast, amino acid biosynthesis genes were

not upregulated in the presence of NONOate, arguing for macrophage specificity (Fig. 5E).

Bacterial microcolonies incubated with IFN/LPS-activated BMDMs showed a profile that

was a hybrid of the responses to macrophages and to NONOate (Fig. 5C-F). Peripheral bacteria,

gated for high mCherry expression after exposure to either activated BMDMs or NONOate

displayed high expression levels of both *hmp* and the cysteine-rich protein-encoding

DN756_01035, which is presumably a component of the NO detoxification response (Figs. 5D,

F). In addition, genes encoding Fe-S cluster biosynthesis proteins SufA/B were upregulated

relative to droplet-grown untreated controls in both peripherally localized populations (Fig. 5D,

5F, NONOate-HI). Interestingly, one of the few genes showing similar downregulation in

response to activated BMDMs and NONOate was *frdA,* which encodes the Fe-S protein fumarate

reductase, potentially reducing the size of Fe-S cluster center pools that need to be rejuvenated

after NO attack (Supplemental Dataset 1). Most notably, there was a class of transcripts only

induced in the presence of IFNγ/LPS-activated BMDMs and not by the other treatments. These

included prophage-associated genes and a predicted ABC regulated efflux pump (*ybhF*,

DN756_13695). Of these, induction of *ccl* (DN756_11195) was observed, which encodes a

protein involved in degrading itaconate, a metabolite produced by macrophages in response to

IFN treatment (Fig. 5D) (37).

When the response of the peripherally-localized bacteria to activated BMDM was

compared to untreated macrophages, the unique set of genes upregulated in response to

macrophage products comes into better focus (Fig. 6A; Supplemental Dataset 2). These included

genes associated with protection from RNS (*tehB* (38)), and Fe-S cluster repair (*ytfE*) (39, 40),

further indicating that NO is the primary driver of the response to activated macrophages. The

lack of arginine starvation in the peripherally-localized bacteria noted above is supported by the

lower expression of arginine biosynthesis-encoding genes (*carAB*, *argB*) relative to nonactivated

controls (Fig. 6A), perhaps because activated macrophages export excess arginine that is

synthesized as a precursor of NO (41, 42). Finally, the specificity of the stress response to NO

was notable.

Regarding genes not clearly associated with the NO response, the peripheral population

showed downregulation of *ompN*, consistent with alterations in membrane integrity or

permeability. In addition, there was induction of a series of genes predicted to encode phage

structural proteins, perhaps due to induction of a *Yptb* prophage (Fig. 6B,C; compare HI gate to

BMDM). Although prophages are known to be induced by DNA damage (43), there was no

evidence for an SOS response preferentially in the HI gate (Supplemental Dataset 3).

Furthermore, treatment with NONOate alone failed to result in induction of most of these genes

(Fig. 6B,C; Supplemental Dataset 3). Therefore, a combination of macrophage exposure and NO

production is associated with a noncanonical stress response that can activate prophage

expression in these microcolonies. Together, these results indicate that NO is the predominant

antibacterial activity produced by activated BMDMs located distant from microcolonies.

The macrophage secreted metabolite itaconate is sensed by microcolonies

The induction of *ccl* was the most notable unexpected regulatory effect of exposure to activated macrophages (Fig. 5C,D). This gene encodes the first step in the pathway for degrading itaconate, a dicarboxylic acid produced by the interferon-regulated immunoresponsive gene 1 (*Irg1*) (37, 44), which has antimicrobial effects on several intracellular pathogens, especially in acidic conditions (45-47). *Yersinia* species are among a subset of bacteria that can degrade itaconate to bypass toxicity (48) by encoding a three-gene operon (Fig. 6D), which breaks down itaconate to pyruvate and acetyl-CoA, and is regulated by ItcR (48). Degradation of itaconate is necessary for *Y. pestis* survival in activated macrophages, but a role for restricting extracellular *Yersinia* within mammalian tissues has not been established (49).

As itaconate may drive differential expression of *Yptb* genes in the presence of IFN/LPS-activated BMDMs, we determined if upregulation of *Yptb* genes in the peripheral HI-mCherry population (Fig. 6C) could be a consequence of itaconate sensing (Fig. 6E). *Yptb* was cultured in triplicate in the presence or absence of itaconate using 3 different pH conditions, and RNAseq analysis was performed on the samples. Each of the three genes in the itaconate degradation operon (*ccl, icl, itc*) were upregulated in response to itaconate, but only at pH=5.2 conditions (Fig. 6E), as observed previously for itaconate signaling in multiple organisms (46, 48, 50). Except for DN756_13955, encoding a putative phage baseplate protein (Fig. 6B), there was no evidence for itaconate regulation of the genes upregulated in the presence of activated macrophages (Fig. 6E). Therefore, itaconate regulation appeared limited to the *ccl* cluster, while induction of prophage-related genes appeared to be a response to other stresses caused by IFN activated macrophages located in the vicinity of the microcolony.

Plasmid-based transcriptional reporters for itaconate degradation were constructed to

analyze the dynamics of the itaconate response. These contained the intact *itcR* regulatory gene

and a gene encoding a fluorescent protein controlled by the *ccl* promoter (Fig. 7A; Table 1) (51,

52). WT *Yptb* (Fig. 7) or a Δ*ccl* derivative (Supplemental Fig. S2) harboring the P_{ccl}::*gfp* plasmid

were grown in acidic conditions at pH = 6.0 or 5.2 (Fig. 7B, C), and fluorescence intensity was

continuously monitored at 26°C. The addition of itaconate had little effect on the growth of *Yptb*

at any pH, with post-exponential phase yields matching or exceeding those observed in the

absence of itaconate (Fig. 7B). Consistent with itaconate control of similar operons (46, 48, 50),

activation of the *ccl* promoter (P_{ccl}) required low pH, with concentrations of 1.0 mM in broth

adjusted to pH=5.2 providing near maximal induction when assayed in late exponential phase

(Fig. 7C; 6 hr timepoint). When challenged with itaconate at pH=6.0, induction of the P_{ccl}::*gfp*

reporter required a high itaconate concentration (5.0 mM) and yielded lower maximal induction

levels than observed at pH=5.2. Notably, peak induction occurred as the bacteria approached

post-exponential phase, but there was little accumulation afterward, in contrast to the results at

pH=5.2, where accumulation continued during post-exponential phase (Fig. 7C).

As acidic conditions were necessary to observe induction of the P_{ccl} reporter, we

investigated if itaconate penetrates a *Yptb* microcolony during infection using the fluorescent

reporter system. To this end, mice were injected intravenously with 10^{3} *Yptb gfp+* harboring

ItcR-Pccl:mcherry itaconate reporter. Three days post infection, spleens were removed and

processed for fluorescence microscopy. The majority of microcolonies did not sense itaconate,

however, each mouse had a rare microcolony showing induction. As itaconate is an exclusively

macrophage-generated product, we determined if induction could result from rare close

encounter with macrophages by probing tissues with α-CD68 (macrophage marker) followed by

fluorescence microscopy. Macrophage-associated *Yptb* showed expression of the itaconate

reporter (Fig. 7E), arguing that itaconate can penetrate a microcolony only after rare direct

contact with macrophages in the tissue microenvironment.

The itaconate degradation operon and microcolony formation during *Yptb* growth in deep

tissue sites.

We predicted that neutrophils surrounding microcolonies may serve as a buffer layer

preventing high levels of macrophage-secreted itaconate from penetrating the microcolony. To

determine if the itaconate degradation operon contributes to survival of *Yptb* during infection,

and if neutrophils buffer from macrophage attack, mice were inoculated intravenously with *Yptb*

WT(pGFP$^+$) or *Δccl-ich-ict* (pGFP$^+$) in the presence or absence of neutrophil depletion.

Neutrophil depletion should result in macrophages directly contacting each microcolony and

increasing itaconate exposure. Three days post-infection, spleens were harvested to quantify

CFU and visualize microcolonies using fluorescence microscopy. Challenge with the *Yptb*Δ(*ccl-*

ich-ict) strain resulted in a small decrease in CFU relative to WT in mock-depleted mice (Fig.

8A). Neutrophil depletion had little effect on this result and, in fact, any differences between WT

and the Δ(*ccl-ich-ict*) strain did not rise to the level of significance (Fig. 8A,C; Mann-Whitney

test). Microcolony size was then determined by image analysis of sections, using GFP

fluorescence (Fig. 8E). There was a small shift downward in microcolony size of the (*ccl-ich-*

ict) strain after neutrophil depletion, which was not observed in the mock-depleted animals (Fig.

8B,D). Although specific for the absence of neutrophils, the shift was not significant based on

multiple statistical tests (unpaired t test; Mann-Whitney).

We noted that microcolonies formed by *Yptb Δccl-ich-ict* appeared to have aberrant

morphology and, in general, lacked rod shape, indicating a stress response (Fig. 8F; examples in

Supplemental Fig. 3).   Interestingly, the bloated bacteria appeared to be in contact with

macrophages (Fig. 8F). We then analyzed images of both WT and *Δccl-ich-ict* after challenge of

either neutrophil-replete or depleted animals. Evidence of bloated bacteria was not correlated

with either the presence of neutrophils or the loss of the itaconate degradation pathway

(Supplemental Fig. 3). Rather, colonies containing 20 or fewer bacteria were often found to

harbor bloated organisms, consistent with disrupted colony formation being associated with

aberrant bacterial morphology. Samples enriched for smaller colonies, therefore, are more likely

to accumulate bloated bacterial cells. These results point to the model that extracellular growth

largely tolerates the absence of the itaconate degradation pathway even when in contact with

macrophages in tissues.

Discussion.

Invasive bacterial pathogens spread to distant tissue sites and establish areas where they

can multiply to high numbers despite the presence of host innate immune cells. Growth within

tissues is followed by the recruitment of neutrophils, macrophages, and inflammatory

monocytes. These inflammatory sites can lead to granulomas or abscesses when immune cells

fail to clear the infection (4, 5). There are few culture models that accurately replicate the

complex structure of pathogen and host immune cells growing in tissue. To address this gap, we

previously developed a 3D model to reconstruct *Yptb* inflammatory sites (16).

During *Yptb* spread into the murine spleen, neutrophils are unable to limit the bacterial

burden, leading to recruitment of macrophages and other inflammatory monocytes to the outside

of the neutrophil layer (16). Our microfluidics-based droplet strategy closely models this second

step in the process, in which macrophages are called to respond to the absence of bacterial

clearance. We view the gel microdroplet as topologically mimicking the zone between the

microcolony and the macrophages, which is inhabited by neutrophils during the formation of a

Yptb microcolony in tissues (7). In support of this hypothesis, both the NO generator DETA-

NONOate and activated BMDMs drove a bacterial transcriptional response that was

morphologically indistinguishable from that observed in tissues (Fig. 3) (7). The ability of

macrophages in their activated state to modulate bacterial gene expression is likely due to the

production of soluble antimicrobial mediators because there is no direct contact with the *Yptb*

microcolony. There are a variety of small secretory products of macrophages that have been

identified, but the roles of these products in controlling bacterial growth or modulating gene

expression of microorganisms are greatly understudied (53).

To complete RNA-seq on *Yptb* subpopulations, the previously developed agarose-

containing gel system had to be reworked and replaced with alginate as the droplet matrix. Both

the microcolony morphology and the spatial regulation of the *Phmp* reporter in response to either

DETA-NONOate or activated BMDMs were similar to that observed in agarose droplets. The

use of this strategy provided immediate benefits and enabled estimation of the amount of RNI

generated by activated macrophages surrounding the microcolonies. Based on the flow

cytometry data of the *hmp* reporter under the two conditions, the amount of RNI produced by

activated BMDMs, which is presumably similar to that observed in tissues, is higher than that

produced by 1 mM DETA-NONOate,.

We hypothesized that *Yptb* growing in the presence of macrophages generates peripheral

and central subpopulations that have distinct transcriptional profiles. RNA-seq analysis revealed

that the bacterial subpopulation with high *Phmp* reporter firing showed a response that was

largely a hybrid of that observed after droplet contact with macrophages and that observed after

exposure to DETA-NONOate. In addition to Hmp induction, genes associated with iron-cluster

formation as well as a poorly annotated cysteine-rich protein that could act as a sink for toxic

RNI- byproducts were observed in the presence of both DETA-NONOate and activated

macrophage adherence. This indicates that NO is the dominant factor produced by activated

BMDMs that target *Yptb* from a distance. Hmp detoxifies NO into nitrate, so we might have

expected that Hmp would generate a considerable pool of nitrate. There was no evidence that

Yptb utilizes nitrate as an alternative electron acceptor despite the availability of this source, with

no induction of nitrate reductase (Fig. 5; Supplemental Dataset 1). This is consistent with oxygen

not being limited and remaining the preferred electron acceptor. There was similar lack of

evidence for alternate electron acceptors during microcolony growth in the murine spleen (7).

There were two notable additions to this hybrid response, involving the induction of the

itaconate degradation pathway (*ccl*) and prophage genes. Although not specific for the peripheral

subpopulation (Fig. 5D), *ccl* upregulation did require macrophage activation by LPS/IFNγ,

presumably in response to liberation of itaconate, which is synthesized within macrophages by

the well-characterized interferon-upregulated Irg1 gene. The encoded enzyme catalyzes the

conversion of the TCA cycle product aconitate to the dicarboxylate itaconate (37). Originally

thought to solely operate as a down modulator of the macrophage inflammatory response (37),

the discovery that the *Y. pestis ccl* cluster encoding itaconate degradation is required for

intracellular survival within activated macrophages indicates that the metabolite plays a role in

restricting intracellular bacterial growth of this closely related pathogen (48, 49). Work in *L.*

pneumophila (54, 55) and *S. typhimurium* (50) supports the concept for restriction of intracellular

bacteria, particularly in acidic environments. There is also evidence that itaconate liberated by

activated macrophages is sensed by extracellular pathogens, such as *Pseudomonas aeruginosa*,

resulting in membrane stress to the microbe (56). Furthermore, degradation of itaconate is

associated with successful persistent infection by *Pseudomonas* (48, 56).

Intravenous infections with a *Yptb* strain deleted for the *ccl-ich-ict* operon argued that

itaconate degradation plays, at best, a subtle role in supporting *Yptb* proliferation in deep tissue

sites. There were small reductions in bacterial yields and microcolony size within spleen tissues,

and there was only rare firing of a *ccl-mcherry* reporter in tissue microcolonies. Those rare

microcolonies did appear to directly abut macrophages, indicating that transcription of the

itaconate degradation operon was linked to close contact with NO-producing cells (Fig. 8).

Given the role of acidic growth conditions both in activating the operon and restricting growth in

the presence of itaconate, it seems unlikely that itaconate could contribute to effective growth

restriction extracellularly. Rather, the most likely route for growth restriction by itaconate would

be targeting of rare, internalized bacteria within an acidic compartment in activated

macrophages, similar to that observed for *Y. pestis* (49). Induction of the operon by extracellular

itaconate could provide *Yptb* with a preparatory induction signal that allows protection once the

bacteria are internalized. As the *Yptb* type III secretion system primarily acts to prevent

phagocytic internalization of the bacteria, this strategy presumably acts as a back-up system to

protect bacteria that fail to block internalization.

The second notable addition to the macrophage/NO hybrid response was the activation of

prophage-associated transcripts after exposure to activated macrophage (Fig. 6). There was clear

spatial regulation to these transcripts, as they were found primarily associated with the

peripherally localized bacterial population. Prophage induction is usually associated with stress

responses to DNA damage or reactive oxygen species (ROS) (57). There was little evidence of a

transcriptional response to ROS or DNA damage, or signatures of an SOS response (43), while

the exposure of bacteria to DETA-NONOate or itaconate alone was insufficient to reproduce the

prophage transcript activation (Fig. 6D). We think it possible the combination of byproducts

generated by macrophage metabolism and RNI may cause some low-level stress response that

results in prophage induction and mimics something akin to a DNA-damage response.

Overall, the alginate droplet system is a powerful technique that allows us to analyze

bacterial subpopulations within a microcolony and identify additional macrophage-secreted

mediators that target *Yptb* (Fig. 9). RNA-seq revealed that there is a *Yptb* subpopulation within

microcolony undergoing a specific stress response resulting in prophage induction (Fig. 9).

Furthermore, the itaconate degradation operon, *ccl-ich-ict,* was upregulated throughout the

colony exposed to activated BMDMs, indicating that itaconate can penetrate these bacteria (Fig.

9). Future work will involve incorporating neutrophils to allow the construction of a complete

inflammatory site that recapitulates tissue behavior. This emphasizes the advantages of using

alginate as a matrix, because the polymer is easily tunable via alginate lyase, allowing control

over the size of the matrix surrounding the microcolony. Theoretically, neutrophils can be

brought into contact with microcolonies by controlled degradation (58) and, furthermore, this

microfluidic approach could serve as a blueprint for dissecting spatial transcriptomics and

complex host-pathogen interactions in a number of other bacterial pathogens

Materials and Methods

Bacterial strains & growth conditions. Plasmids used are described in Table 1. All

experiments used derivatives of the *Y. pseudotuberculosis* strain IP2666 (Table 2; (59)). For all

droplet experiments, bacteria were grown overnight into stationary phase in 2xYT broth (60); (7)

at 26 $^{\circ}$C with rotation. For itaconate (Sigma # I29204-100G) exposure in broth cultures,

overnight cultures were diluted 1:100 in LB and rotated at 37 $^{\circ}$C for 4 hours in the presence of

noted concentrations of itaconate.

Generation of plasmid-based reporter strains. The *Y. pseudotuberculosis P$_{hmp}$::mCherry*

reporter strain in this study has been previously described (60); (7). GFP^{+} strains encode GFP

under the control of the unrepressed *P$_{tet}$* promoter of pACYC184 (61). The *P$_{hmp}$::mCherry*

transcriptional fusion was constructed by fusing the *hmp* promoter to *mCherry* using overlap

extension PCR, and cloning into the pMMB67EH plasmid (62), Strains that harbor both

reporters are co-transformants of the compatible pACYC184 and pMMB67EH plasmids. The

ItcR-P$_{ccl}$::mcherry and *ItcR-P$_{ccl}$::gfp* transcriptional fusions were constructed by fusing *ItcR- ccl*

promoter to *mCherry* and *gfp* using Gibson Assembly$^{®}$ (New England BioLabs) and cloning into

pMMB67EH and pACYC184, respectively (51, 52).

Generation of ccl-ich-ict mutant strains. Strains having deletions in the gene of interest

were generated by amplifying the start codon + 3 downstream codons, the 3' terminal 3 codons +

the stop codon and fusing these fragments to generate a 24 base pair scar sequence. Deletion

constructs were amplified with 500 base pairs flanking sequence on each side, cloned into the

suicide vector, pSR47s (63, 64), transformed into DH5α λ*pir* and mated into *Y.*

pseudotuberculosis IP2666 using the helper strain RK600 (60). Sucrose counterselection was

used to isolate strains that had recombined the *ccl-ich-ict* construct and removed the WT copy of

the gene, then strains were confirmed by PCR and sequencing of PCR product.

Droplet generation. CAD-designed microfluidics chips having 38 independent devices

controlled by two ports were constructed according to published protocols (65) at the Boston

College Nanofabrication Facility (https://www.bc.edu/bc-

web/schools/mcas/sites/cleanroom.html). Prior to droplet generation, the device was primed with

fluorinated oil (3M Novec 7500) and connected to two programmable syringe pumps (Harvard

Apparatus 11 Elite Series) located in a temperature-controlled room at 37 ^{o}C. One pump was

fitted with a 1ml syringe (BD #309628) having a 27-gauge needle (BD #305109), which was

filled with 1.5% Pico-Surf 1 (Dolomite Microfluidics, 5% in Novec 7500) in Novec 7500 oil

(16). In parallel, overnight cultures were diluted 1:2000 in 1% Na-alginate (Fisher

#AC177772500) in DMEM with glutamine, 10% FBS, 25mM Hepes and 25 mM CaCO$_3$

nanoparticles (SSNano #1951RH) containing RGD-alginate at a concentration that was 5% of

unmodified Na-alginate (Novamatrix, Novatach MVG GRGDSP), yielding approximately 5 x

10^6 cells/ml prior to droplet generation. The mix was loaded into a 1 ml syringe that was fitted

onto a second pump. Polyethylene (PE/2) tubing (SCI COM #BB31695-PE/2) was attached to

each syringe and then inserted into the appropriate ports on the microfluidics device (16). The

pump holding the oil-phase was set to 500 μl/hour while the other pump was set to 200 μl/hour

and droplets were collected in a 1.5 ml microfuge tube through tubing that had been inserted into

the droplet collection port.

Oil Removal. 100 μl droplets was introduced into a 1.5 ml microfuge tube and 400 μl of

1.5% Pico-Surf 1 in Novec 7500 oil was added to the droplets. To crosslink the alginate, 500 μl

of 0.1% acetic acid in Novec 7500 oil was added to the droplets. After mixing by inverting the

tube several times, the dilute acetic acid in oil was removed from the tube and 500 μl of 10%

1H,1H,2H,2H-Perfluoro-1-octanol (PFO) (Sigma #370533-25G) in Novec 7500 was added to the

droplets. After mixing vigorously for approximately 10 seconds, 500 μl of DMEM with 10%

FBS was added and pipetted up and down to resuspend the droplets without disturbing the oil

layer. The DMEM/droplet layer was transferred to a new 1.5 ml microfuge tube, subjected to

centrifugation for 30 seconds at 250 RCF, and washed with DMEM. The remaining droplets

were washed and resuspended in 2xYT broth supplemented with 5 mM $CaCl_2$ to maintain

alginate droplet integrity.

Droplet dispersal. Droplets were washed once in DMEM at the indicated timepoints, and 900 μl

of RNA*later* (Invitrogen #AM7021) was added to the samples dispersing droplets. Samples were

incubated overnight at 4^oC, then resuspended with 10 mL ice cold PBS. Samples were centrifuged at

5,000 x *g* for 15 minutes. The supernatant was removed and the bacterial pellets were resuspended in 500

μl of PBS sample, then prepared for flow analysis.

Analysis of the nitric oxide response. To analyze the response of colonies to exogenous

NO, droplets containing *Y. pseudotuberculosis* were generated, oil was removed, and the droplets

were rotated for 7 hours at $26\,^o$C in 2xYT broth to allow colony formation. After the 7-hour

growth period, droplets were subjected to centrifugation for 30 seconds at 250 RCF and washed

twice in DMEM. 50 μl of droplets were resuspended in 1 ml DMEM with glutamine (Gibco)

supplemented with 10% FBS in 12- well non-tissue culture treated plates and exposed to DETA-

NONOate (Sigma #AC32865) during growth at 37 °C with 5% CO_2 for the indicated times.

Analysis of the itaconate response. For each condition, 3 independent cultures were

made from single colonies and grown overnight in LB broth with aeration at 26^oC. The bacteria

were then diluted 1:100 in LB broth and grown in same medium to $A_{600} = 0.3$. The bacteria were

pelleted, washed three times in 1X PBS/Mg/Ca, and diluted 1:30 into DMEM/25 mM HEPES +

10% FBS equilibrated to noted pH levels in the presence or absence of 1 mM itaconate and

prewarmed to 37^oC. Bacteria were grown to $A_{600} = 0.1$ and 50% of the cultures were pelleted and

flash-frozen in liquid N_2. The rest of the cultures were grown to $A_{600} = 0.3$, pelleted and flash-

frozen in liquid N_2. RNA was extracted and processed for RNA-tagseq analysis.

Isolation of bone marrow-derived macrophages. Bone marrow cells were isolated from

femurs, tibias, and humeri of female 6-8-week-old C57BL/6 mice, and terminally differentiated

into macrophages in medium containing mouse CSF generated as previously described (16, 66).

Bone marrow-derived macrophages (BMDMs) were subsequently frozen in fetal bovine serum

(FBS) with 10% dimethyl sulfoxide (DMSO) and plated one day prior to incubation with

droplets in 90% fresh medium (DMEM with glutamine (Gibco) supplemented with 10% FBS)

spiked with 10% sterile medium containing macrophage colony stimulating factor (MCSF) in

5% CO_2 at $37^{\circ}C$. Medium containing MCSF was conditioned by growth in the presence of

mouse 3T3 fibroblasts that overproduce mouse MCSF at 37 °C with 5% CO2, as previously

described (66) (67). To stimulate BMDMs to induce iNOS expression, cells from frozen stocks

were plated on non-tissue culture treated plates and allowed to recover overnight. The following

day, BMDMs were stimulated with 200 U/ml IFNγ (Peprotech) and 100 ng/ml LPS (Sigma) for

12-15 hours (16, 33).

Droplet-BMDM experiments. Droplet-BMDM experiments were completed as

previously described (16). Droplets containing *Y. pseudotuberculosis* were generated, oil was

removed, and the droplet-encased bacteria were grown with aeration for 7 hours at $26^{\circ}C$ in

2xYT broth. After the 7-hour growth period, droplets were pelleted for 30 seconds at 250 RCF,

washed twice in DMEM supplemented with 10% FBS and supernatant was removed. 50 µl of

droplets were transferred to a well in a 12-well non-tissue culture treated plate, approximately 2

x 10^6 BMDMs in DMEM with 10% FBS were added to the well, and the incubation proceeded at

37 °C in the presence of 5% CO_2 for 4 hours. Samples were fixed in 2% paraformaldehyde

(PFA) in PBS, washed in PBS, and fluorescent reporters were visualized by microscopic

observation.

Murine model of systemic infection. Six to 8-week old female C57BL/6 mice obtained

from Jackson Laboratories (Bar Harbor, ME) were injected intravenously with 10^3 bacteria.

Three days post inoculation, spleens were removed and processed as described previously (7,

60). For neutrophil depletions, mice were intraperitoneally injected with 50 μg rat anti-mouse

Ly6G antibody (BD Biosciences # 551459) 16 hours prior to and 24 hours post-infection (32).

All animal studies were approved by the Institutional Animal Care and Use Committee of Tufts

University.

Flow cytometry and fluorescence-activated cell sorting (FACS). After microcolony dispersal,

samples were filtered through 40 μm filters. Filtered samples were added to 5ml FACS tubes containing 2

ml PBS on ice. Fluorescent reporters were detected using a Bio-Rad S3e cell sorter. Purity Sort Mode was

used during sorting, and the priority was set to the high *mCherry* subpopulation when applicable. Samples

were sorted into 5 ml tubes containing 0.5ml ice cold PBS. 3-10 x10^6 events were collected for each

sample. After sorting, samples were subjected to centrifugation 5,000 x g for 15 min at 4°C, supernatants

were removed, and pellets were overlayed with 400 uL of ice cold PBS. Samples were flash frozen in

liquid nitrogen and stored at -80°C for later use.

RNA extraction. 200 μl of pre-heated (95 $^\circ$C) Max Bacterial Enhancement reagent

(Ambion #16122-012) was added to frozen bacterial pellets. Afterward, samples were transferred

to a 1.5 ml microfuge tube and incubated at 95 $^\circ$C for 5 minutes. Extraction of total RNA was

conducted using the Zymo Direct-Zol Microprep Kit and following the protocol for cells in

suspension (Zymo #R2026).

RNA-seq sample preparation. cDNA libraries were generated following the RNAtag-seq

protocol as previously described (68, 69), but ribosomal RNA (rRNA) was depleted using Illumina®

Ribo-Zero Plus rRNA depletion kit to deplete prokaryotic rRNA and any contaminating eukaryotic

rRNA. Libraries were sequenced at High-Output for 75 cycles on Illumina NextSeq500 (TUCF Core).

Computational Analysis. RNA-Seq data was analyzed using customized pipeline. In

brief, raw reads were demultiplexed by 5' and 3' indices and mapped to the reference genomes

(CP032566.1 and CP032567.1) using bwa mem [PMID: 19451168]. Mapped reads were

aggregated by featureCount [PMID: 24227677] and differential expression was calculated with

DESeq2 (36, 70). *apeglm* estimation [https://doi.org/10.1093/bioinformatics/bty895] was used

within the lfcShrink function. In each pair-wise differential expression comparison, significant

differential expression was filtered based on svalue < 0.01. Hierarchical cluster analysis was

performed for the top 50 genes having the most significant differential expression.

Fluorescence microscopy. After 4 hours of nitric oxide exposure or BMDM challenge,

droplets were fixed in 2% PFA in PBS for 10 minutes. Droplets were visualized by transferring

the sample to a microscope slide and covering with a coverslip. To analyze tissue samples*,*

C57BL/6 mice were inoculated intravenously with the indicated strains. Three days post

inoculation, spleens were harvested and fixed in 4% PFA in PBS for 3 hours, tissue was frozen-

embedded in Sub Xero freezing media (Mercedes Medical) and cut into 10 μm sections using a

cryostat microtome (Microm HM505E). To visualize reporters, sections were thawed in PBS,

stained with Hoechst at a 1:10,000 dilution, washed in PBS, and coverslips were mounted using

ProLong Gold (Life Technologies). For immunofluorescence staining of macrophages, sections

were thawed, blocked with 2% BSA, permeabilized with PBT (0.2% Triton X-100, 0.1% BSA),

and stained using a 1:1000 dilution of Alexa Fluor® 594 anti-mouse CD68 antibody (Biolegend #

137020). Tissue was imaged with a 63x objective on a Zeiss Axio Observer.Z1 (Zeiss)

microscope with Colibri.2 LED light source, an Apotome.2 (Zeiss) for optical sectioning, and an

ORCA-R^2 digital CCD camera (Hamamatsu) (7, 60); (16)}.

Determination of microcolony area. To determine microcolony area in tissue, images

were captured by fluorescence microscopy using a 20x lens with analysis in Volocity^{TM} as

previously described (16). Microcolony area was determined by identifying a threshold that

defines edges of microcolonies in the GFP channel and determining the number of pixels in the

region of interest (ROI). The data were then converted to metric scale using a stage micrometer

and displayed as μm^2.

Quantitative image analysis. MATLAB scripts were written to calculate the average

intensity of fluorescence about the periphery of microcolonies and point of lowest fluorescence

as previously described (16); copy archived at https://github.com/elifesciences-

publications/droplet.

Deposition of RNA-seq data. All raw RNA-seq datasets have been deposited in SRA

under BioProject PRJNA1120393.

Acknowledgements.

We would like to thank Dr. Albert Tai and members of the Tufts University Core Facility

(TUCF) Genomics unit for instruction, help, and guidance throughout this work, as well as Mr.

Steve Kwok of the Tufts Thorley-Lawson Sorting Facility for hands-on help with these studies.

We thank Drs. Bixi He, Kevin Manera, Atish Roy Chowdhury, and Efrat Hamami for review of

the manuscript. The work in this study was supported by NIAID grants R01AI110684 and R21

151593 to RRI and U01AI124302 and R01AI110724 to TvO. SC was supported by predoctoral

training grant T32 TM007310 from NIGMS

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Figure Legends

Figure 1. *Yersinia pseudotuberculosis* grows as a microcolony in microfluidics-driven

alginate droplets. (A). Construction of alginate droplets. 1) Molten alginate, of which 5% is

RGD-linked, and 25 mM $CaCO_3$ nanoparticles were introduced into a microfluidics device,

allowing encapsulation within oil-coated droplets. 2) Acetic acid (final concentration 0.05%) was

added to alginate-$CaCO_3$ droplets in oil to allow for gelation of alginate. Protons diffuse into the

alginate-$CaCO_3$ droplets in oil. 3) H^+ ions exchange with Ca^{2+} ions, releasing CO_2. 4) Free Ca^{2+}

binds alginate and crosslinks the matrix. (B). Image of alginate droplets with oil removed after

in-oil polymerization of alginate. Scale bar: 50μm. (C). *Y. pseudotuberculosis* grows as a

microcolony in droplets. Droplets containing encapsulated *Y. pseudotuberculosis gfp+* were

cultured overnight at 26°C, and microcolonies were visualized by brightfield and fluorescence

microscopy. Dashes: define outline of droplet. Arrows: edge of droplets. Scale bar: 20μm.

Figure 2. Spatial regulation of *hmp* expression in tissues can be reproduced in alginate

droplet culture. (A). Experimental overview. Droplets seeded with *Y. pseudotuberculosis*

harboring fluorescence reporters were incubated overnight at $26\ ^{o}$C and 1 mM DETA-NONOate

was added to the cultures for 4 hours at $37\ ^{o}$C, 5% CO_2. After 4 hours, the droplets were fixed

and visualized using brightfield and fluorescence microscopy. (B). Representative WT

microcolony after 4 hours of treatment with 1mM DETA-NONOate. (C). Periphery versus PLI

ratio for microcolonies as a function of DETA-NONOate concentration in microcolonies. Gray

circles: 105 individual microcolonies measured for each condition from 3 biological replicates.

Lines indicate the medians. Statistics: Mann-Whitney test, ****$p<0.0001$.

Figure 3. Activated BMDMs drive peripheral expression of *hmp* in alginate droplet

microcolonies. (A). BMDMs adhere to alginate droplets containing 5% RGD-alginate. Droplets

containing *Y. pseudotuberculosis* were grown overnight at 26 °C prior to incubation with

LPS/IFNγ-primed BMDMs for 2 hours at 37 °C, CO_2. The fixed droplets were visualized using

phase contrast and fluorescence microscopy. Scale bars: 50μm. (B). Representative WT

microcolony incubated with LPS/IFNγ-primed BMDMs for 4 hours. Scale bar: 20μm. (C).

Periphery versus PLI ratios for microcolonies incubated with BMDMs treated as noted. Gray

circles: 105 individual microcolonies from each condition from 3 biological replicates. Lines

indicate the median. Statistics: Mann-Whitney test, ****$p<0.0001$. (D). LPS/IFNγ-primed

BMDMs secrete more NO than 1 mM DETA-NONOate. Flow cytometry of dispersed

microcolonies after 4 hours of incubation with DETA-NONOate or BMDMs. Dispersed bacteria

were gated for GFP[+] and the median mCherry fluorescence intensity was determined from 6-7

biological replicates (Materials and Methods). Lines represent geometric mean with 95% CI.

Statistics: Unpaired parametric t test, assuming both populations have the same SD,

****$p<0.0001$, **$p<0.01$.

Figure 4. Flow sorting of *Yersinia pseudotuberculosis* subpopulations originating from alginate

droplets. (A). Experimental overview. WT *Yptb gfp+, $P_{hmp::mcherry}$* was encapsulated into alginate

droplets and incubated for 7 hrs in culture medium to allow formation of microcolonies. The droplets

were then incubated with either DETA-NONOate or BMDMs for 4 hrs, and RNA*later* was added to the

droplet-encompassed microcolonies. After subsequent extraction of *Y. pseudotuberculosis*, the bacterial

subpopulations were sorted by flow cytometry into peripheral *hmp*[+] mCherry(HI) and central *hmp*[-]

mCherry (LO) populations. (B-E) Flow cytometry of *Yptb* from droplets incubated with: (B) 0 mM

DETA-NONOate; (C) 1 mM DETA-NONOate; (D) BMDMs; or (E) LPS/IFNγ-primed BMDMs. (B-E)

Green rectangular gates: GFP+ events. (C,E) Red rectangular gates: mCherry-HI (hmp^+) events. Black

rectangular gates: mCherry-LO (hmp^-) events. Flow was performed using 488nm (GFP) and 561 nm

(mCherry) laser excitation, with GFP emission detected at 510-540 nm and mCherry detected at 602-627

nm emission windows.

Figure 5. *Yptb* originating from the high *Phmp::mCherry* gate are transcriptionally distinct from the

rest of the bacterial population. *Yptb* was grown in droplets (Materials and Methods), exposed to noted

conditions, RNA was isolated from sorted gates (Fig. 4), and RNA-tagseq analysis was performed using

the noted conditions. (A,C,E) Volcano plots show statistical significance plotted as a function of the

magnitude of differential expression for droplet-grown bacteria, comparing untreated droplets (-BMDM

or -NONOate) to the noted bacteria populations. Red: s-value < 0.01 and log_2-fold change of <-0.58;

Gray: s-value ≥0.01; Green: s-value < 0.01 and log_2-fold change of > 0.58. (B,D,F) Heat maps of log_2 fold

change relative to untreated droplets (-BMDM or -NONOate) are presented for 25-30 of the most highly

upregulated genes when exposed to (B) unstimulated BMDMs, unsorted; (D) LPS/IFNγ-primed BMDMs,

sorted P_{hmp}-*mcherry*-HI; and (F) 1 mM NONOate, sorted for P_{hmp}-*mcherry*-HI. Data are presented for the

following populations: BMDM: exposed to unstimulated macrophages; LPS_Ifn_LO: exposed to

LPS/IFNγ-primed BMDMs and sorted for P_{hmp}- mCherry-LO fluorescence population; LPS_Ifn_HI:

exposed to LPS/IFNγ-primed BMDMs and sorted for the P_{hmp}-mCherry-HI fluorescence population;

NONOate_LO: incubated with 1 mM NONOate and sorted for the P_{hmp}- mCherry-LO low fluorescence

population; NONOate_HI: incubated with 1 mM NONOate and sorted for the P_{hmp}- mCherry-HI

fluorescence population. Sorted populations defined in Fig. 4.

Figure 6. Spatial regulation of the response to activated BMDM. (A) Transcriptional response of *Yptb*

located in the P_{hmp}-*mcherry* high fluorescence gate (HI) compared to that after incubation of droplets with

unstimulated BMDMs. The statistical significance plotted as a function of the magnitude of differential

expression of the bacteria isolated from the two gates. Red: s-value < 0.01 and log_2-fold change of <-0.58;

Gray: s-value ≥0.01; Green: s-value < 0.01 and $\log_2$-fold change of > 0.58. (B) Genetic map of cryptic

phage locus harboring genes differentially upregulated in the P_{hmp}-*mcherry* high fluorescence gate (HI)

compared to untreated droplet-grown bacteria. (C). Heat map of the 29 transcripts, chosen based on their

being the most highly differentially-transcribed genes comparing P_{hmp}-*mcherry* high fluorescence gate

(HI) to P_{hmp}-*mcherry* low fluorescence gate (LO). BMDM: transcript expression after incubation with

unstimulated macrophages compared to no macrophage incubation; LPS_Ifn_LO: exposed to LPS/IFNγ-

primed BMDMs and sorted for P_{hmp}-*mcherry*-LO population, compared to no macrophage incubation;

LPS_Ifn_HI: exposed to LPS/IFNγ-primed BMDMs and sorted for the P_{hmp}-*mcherry*-HI population

compared to no macrophage incubation; NONOate_LO: incubated with 1 mM NONOate and sorted for

the P_{hmp}-*mcherry*-LO population compared to no NONOate incubation; NONOate_HI: incubated with 1

mM NONOate and sorted for the P_{hmp}-*mcherry*-HI population compared to no NONOate incubation.

Scale: $\log_2$ differences displayed. Sorted populations defined in Fig. 4. (D). Genetic map of itaconate

degradation operon and ItcR regulator. (E). Phage genes are not induced by itaconate. *Yptb* was grown in

broth and exposed to 1.0 mM itaconate as described at pH 5.2, 6.0, and 7.0 with $\log_2$ fold change shown

compared to absence of NONOate at noted pH. Genes chosen were from panel B. Top Panel: *ccl* operon,

the other most differentially regulated genes in the bottom panel.

Figure 7. Limited itaconate diffusion in tissues. (A,B). Fluorescent reporter plasmids that

allow sensing of itaconate. Plasmids have intact *itcR* regulator that senses itaconate and controls

ccl promoter. Fusions to *mcherry* and *gfp* were constructed as identical plasmids (Materials and

Methods). (C,D). Overnight broth cultures of *Y. pseudotuberculosis* containing the P_{ccl}-*gfp*

reporter were diluted to A_{600}=0.03 in LB adjusted to either pH=5.2 or pH=6.0 in the presence of

noted itaconate concentrations. Increased mass was read at A_{600} (C), while fluorescence of the

GFP reporter was measured excitation = 485/9 nm and emission = 507/9 nm (D). (E,F) C57BL/6

mice were injected intravenously with 10^3 WT *gfp+* harboring the *itcR-p_{ccl}:mcherry* reporter and

spleens were harvested 3 days post inoculation. Spleen tissue was probed with α-CD68 (blue)

and visualized by fluorescence microscopy. Scale bars: 10 μm.

Figure 8. Effects of loss of itaconate degradation operon during tissue growth of *Yptb*.

C57BL/6 mice were intraperitoneally injected with α-Ly6G (clone 1A8) or PBS 16 hours prior to

and 24 hours post-infection to deplete or perform mock-depletion of neutrophils (PMN). Mice

were inoculated intravenously (i.v.) with 10^3 WT *gfp+* or *Δccl-ich-ict gfp+* bacteria, and spleens

were harvested 3 days post-inoculation (PI) for quantification of CFU and fluorescence

microscopy. Lines represent the median. (A, B): Mock-depleted C57BL/6 mice. (A) Total CFU

and (B) microcolony areas were quantified (Materials and Methods). Lines represent the median.

(C,D): α-Ly6G depleted C57BL/6 mice. (C) Total CFU and (D) microcolony areas were

quantified. Lines represent the median. Blue, red, and gray dots: Individual mice from 3 separate

experiments. Statistics: Mann-Whitney test, **p<0.005, *p<0.05, ns: not significant.

(E,F):Representative WT and *Δccl-ich-ict* microcolonies showing variation in morphology. Scale

bar: 20 μm (E): Representative WT and *Δccl-ich-ict* microcolonies after infection in neutrophil-

depleted mice. Scale bar: 20 μm

Figure 9. Bioengineered droplets allow for the transcriptional analysis of bacterial

subpopulations and inform about the tissue microenvironment. Bacteria are grown *in situ*

within alginate droplets prior to challenge with iNOS$^+$ BMDMs. Activated BMDMs secrete NO,

itaconate, and other metabolites which target a *Yptb* microcolony. Hmp+ bacteria on the

periphery of a microcolony upregulate genes involved in iron sulfur cluster assembly and stress

adaptation. This indicates that the peripheral cells within a microcolony are likely slow-growing.

Supplemental Figure 1. The presence of RNA*later* results in loss of emission from

fluorescent proteins. Overnight broth cultures of *Yptb gfp+ Phmp::mcherry* were back diluted

1:100 in 2xYT and grown in the presence of 2.5 mM DETA-NONOate for 4 hours prior to

applying the agarose/ HyStem®-C Hydrogel droplet dispersal protocol (16) in the presence of

PBS or RNA*later*, and fluorescence was analyzed after each step in the protocol by flow

cytometry. Flow fluorescence profiles are as follows. (1.) Pelleted bacterial cells resuspended in

100 μl PBS or RNA*later* (RNA*later* Treatment); (2.) RNA*later* Treatment in presence of

incubation at 50°C for 3 min. (3.) RNA*later* Treatment in presence of addition of collagenase,

hyaluronidase, and agarase. (4.) RNA*later* Treatment in presence of incubation step 37 ^{o}C for 45

min. (5.) RNA*later* Treatment in presence of incubation step 60 ^{o}C for 3 min.

Supplemental Figure 2. Transcriptional response to itaconate in the absence of the *ccl*

operon. WT and Δ(*ccl-ich-ict*) *Yptb P_{ccl}::gfp* strains were grown at 26°C in DMEM/HEPES +

10% FBS adjusted to pH = 6.0 (blue symbols) or pH = 5.2 (red symbols) in the presence or

absence of various concentrations of itaconate in 96 well plates. Fluorescence of GFP was

continuously monitored (ex=485nm, em=507nm; Materials and Methods) and plotted as a

function of time. (A). Expression of P_{ccl} promoter in WT strain background in response to

noted itaconate concentrations (0.0, 1.0, 2.5 and 5.0 mM in DMEM/HEPES+10% FBS) and pH

conditions as monitored by GFP fluorescence. (B). Expression of P_{ccl} promoter in Δ(*ccl-ich-ict*)

strain background in response to noted itaconate concentrations (.0, 2.5 and 5.0 mM in

DMEM/HEPES+10% FBS) and pH conditions as monitored by GFP fluorescence. (C).

Hyperexpression of the P_{ccl} promoter in the absence of the itaconate degradation pathway in

medium adjusted to pH = 6.0. Data from the 5 hour timepoint from panels A and B (noted by

arrows) were plotted to compare the response of the WT and Δ(*ccl-ich-ict*) strain to itaconate

concentrations. (D). Relative growth inhibition at low pH of *Yptb* strains. WT and Δ(*ccl-ich-ict*)

Yptb P$_{ccl}$::gfp strains were grown at 26°C in DMEM/HEPES+10% FBS medium adjusted to pH

= 5.2 in the presence or absence of noted concentrations of itaconate in 96 well plates. Growth

was monitored by reading absorbance at A$_{600}$ and plotted as a function of time.

Supplemental Figure 3. Small colony size is associated with bloated bacterial morphology.

Shown are colonies from spleen sections qualitatively scored as small and large then imaged for

bacterial morphology. The noted bacterial strains (*Yptb-gfp* WT and Δ(*ccl-ich-ict*)) were

infected as described (Materials and Methods) into C57Bl/6 mice in the absence or presence of

neutrophil depletion with anti-Ly6G. After euthanization, colony sizes were measured (Fig. 8)

and GFP fluorescence imaged.

960
961   **Table 1 Plasmids used in this study.**
962

Fusion	Plasmid backbone	Reference
gfp+ (Ptet::gfp)	pACYC184	(60)
Phmp::mcherry	pMMB67EH	(7)
ItcR-Pccl::mcherry	pMMB67EH	This study

ItcR-Pccl::gfp	pACYC184	This study

969 **Table 2 Strains used in this study.**

Background	Strain	Reference
IP2666	WT *gfp+*	(7)
IP2666	WT *gfp+, Phmp::mcherry*	(7)
IP2666	WT gfp+, *ItcR-Pccl::mcherry*	This study
IP2666	WT *ItcR-Pccl::gfp*	This study
IP2666	*Δccl-ich-ict gfp+*	This study

A.

Oil Phase

Oil Phase + Acetic Acid

●=$CaCO_3$ nanoparticle   〜=Alginate   ●=Ca^{2+}

B.

C.

20um

A.

B.

gfp   hmp   merge   BF   20μm

C.

Periphery vs. PLI Ratio

****

LPS+ IFNγ   −   +

D.

Median Relative Intensity (mCherry)

****

**

****

DETA-NO   −   +   −   −
LPS+ IFNγ   −   −   −   +

BMDMs

Droplets + 0mM DETA-NO

B

GFP+
95.4

GFP

Droplets + 1mM DETA-NO

C

GFP+
93.6

hmp+
10.3

hmp-
10.1

Droplets + BMDMs

D

GFP+
90.5

GFP

Droplets + LPS/IFNγ-BMDMs

E

GFP+
93.4

hmp+
11.1

hmp-
10.9

P_{hmp}::mCherry

P_{hmp}::mCherry