Introduction

Inflammatory bowel diseases (IBD), including Crohn’s disease (CD) and ulcerative colitis (UC), are characterized by chronic inflammation and dysregulated immune responses elicited by dysbiosis of the gut microbiota.\(^1\) Normal wound healing mechanisms respond to injury by replacing damaged cells and excreting ECM to facilitate tissue repair; the repair process depends on the balance between ECM production and degradation.\(^2\) In patients with IBD, bowel injury elicits a chronic inflammatory response, which subsequently triggers fibrogenesis.\(^3\) Following tissue injury, mesenchymal cells (responsible for ECM production) become activated and proliferate due to interactions between paracrine and autocrine factors.\(^2,3\) These profibrotic mediators derive from interactions between the immune system, intestinal microbiota, epithelial cells, ECM, and mesenchymal cells.\(^2\) Fibrosis develops when mesenchymal cells (myofibroblasts, fibroblasts, and smooth muscle cells) become over activated and cause excessive ECM deposition without balanced degradation, resulting in regulatory failure.\(^2,3,4\) Intestinal fibrosis is a major complication associated with IBD; a ramification of chronic inflammation, fibrosis is characterized by excessive extracellular matrix (ECM) deposition and architectural distortion of the colon.\(^2,3\) Fibrosis ultimately affects at least 40% of patients with CD and 5% of patients with UC.\(^3,4\) In many cases, fibrosis results in intestinal stricture formation which lead to bowel obstructions, the main reason for surgical intervention.\(^2,3\) Current treatment options primarily involve anti-inflammatory drugs and surgical interventions, but they are inadequate and recurrent symptoms are common.\(^3,4\) The deficits in therapeutic options are largely due to lack of relevant animal models and insufficient knowledge regarding the cellular and molecular pathways involved in the development of fibrosis.\(^3,4\)

Dysbiosis of the microbiota is largely implicated in the development of IBD, and evidence supports the implication of the gut microbiota in fibrogenesis as well.\(^4\) The IBD microbiome, as a consequence of inflammation, is typically characterized by a decrease in microbial diversity alongside an increase
in aggressive species. One group of bacteria in particular, adherent-invasive *Escherichia coli* (AIEC), is overrepresented in the guts of both humans with IBD and animal models of colitis compared to healthy individuals. AIEC are capable of instigating and propagating chronic intestinal inflammation.

AIEC have several putative virulence factors, one being the yersiniabactin (Ybt) high pathogenicity island (HPI), which is overrepresented in AIEC strains. Many gut resident Enterobacteriaceae contain this HPI, it encodes proteins for the biosynthesis of the siderophore Ybt and its cognate receptor, FyuA. Once secreted by the bacteria, Ybt chelates Fe\(^{3+}\) and other metals for bacterial metal acquisition. Metals like iron and copper are essential for both host and bacterial growth, but bacteria are often subjected to metal-limiting conditions within a mammalian host. This leads to the upregulation of high-affinity metal uptake systems like Ybt-FyuA, a system implicated in both virulence and bacterial fitness. Once secreted Ybt is bound to a metal, FyuA is required for Ybt-metal import by the bacteria. While the Ybt-FyuA system is overrepresented in bacterial strains linked to IBD, its precise role in microbial-induced proinflammatory responses and subsequent disease development is unknown.

We investigated the role of the Ybt-FyuA system in colitis utilizing a gnotobiotic Interleukin-10-deficient (*Il10*\(^{-/-}\)) mouse model, which is an inflammation susceptible germ-free (gf) mouse that will develop spontaneous colitis when colonized with bacteria and introduced to bacterial antigens (Figure 1). Inactivation of the Ybt system had little impact on inflammation and colitis in *Il10*\(^{-/-}\) mice. However, colonization with strains lacking the FyuA receptor, and thus unable to import Ybt, induced intestinal fibrosis. Colonization with strains incapable of Ybt biosynthesis attenuated the fibrotic development. This pathology required Ybt synthesis, but did not impact bacterial colonization and
was independent of Ybt uptake through FyuA, suggesting a novel mechanism for the siderophore system separate from its role in bacterial iron acquisition and fitness. Therefore, this data suggests that the bacterial small molecule Ybt promotes fibrosis in AIEC-colonized Il10−/− mice.

**Figure 1. In vivo Il10−/− (inflammation prone) gnotobiotic colitis model.**

![Diagram showing the experimental setup](image)

**Fig 1.** Germ-free Il10−/− mice are mono-associated with isogenic mutant strains of *E. coli* NC101 deficient in various components of the Ybt-FyuA system. WT mice develop neither inflammation nor fibrosis in this model. FyuA encodes the FyuA receptor, and irp1 is required for Ybt biosynthesis. None of the three mutant strains can import Ybt in our mono-association model. After 10 weeks of colonization, we harvest tissues for histologic and molecular analyses. My role was to evaluate disease in our cohort of ΔfyuA Δirp1 colonized Il10−/− mice.

Two fundamental questions were explored to evaluate the role of the Ybt-FyuA uptake system on IBD-associated fibrosis. To determine whether Ybt was essential for promoting fibrosis in *E. coli* NC101 ΔfyuA (an AIEC strain unable to produce FyuA) mono-associated Il10−/− mice, germ-free mice were colonized with the NC101 ΔfyuA Δirp1 (an AIEC strain unable to produce the FyuA receptor or Ybt siderophore) double-knockout strain. Mice mono-associated with NC101 ΔfyuA Δirp1 phenotypically mirrored mice colonized with NC101 Δirp1 in that both had intestinal inflammation, but no fibrosis. These results supported the hypothesis that Ybt is necessary for the development of the fibrotic phenotype. In order to further examine the molecular and cellular mechanisms implicated in fibrosis, the role of the *E. coli* Ybt-FyuA uptake system on fibroblast (cell type most likely responsible
for excess ECM deposition) activation was evaluated in vitro. Live E. coli NC101 strains missing various components of the Ybt-FyuA system were found incapable of directly stimulating fibroblasts. These negative results lead us to evaluate the activation potential of bacterial small molecules, including Ybt and various other metal chelators, on fibroblasts. Our findings support a model in which metal-chelating small molecules of the microbiota induce inflammation-associated fibrosis through indirect effects on fibroblast activation and subsequent ECM production.

Materials and Methods

Mice: Germ-free (GF) Il10−/− and WT mice of 129S6/SvEV background were originally derived and maintained under GF conditions at the National Gnotobiotic Rodent Resource Center at the University of North Carolina (UNC), Chapel Hill. All procedures were approved by the UNC Institutional Animal Care and Use Committee.

In vivo mono-association of gnotobiotic mice with E. coli NC101 variant strains: An overnight bacterial culture of E. coli NC101 or NC101 ΔfyuA Δirp1 mutant was utilized to monoassociate mice (age 8-11 weeks, n=15) via oral and rectal swab. Mice were maintained in gnotobiotic isolators for 10 weeks. Necropsy was performed at 10 weeks post-colonization. Tissues were collected for histological analyses, and additional tissues were harvested and archived for molecular analyses. Histologic and immunofluorescent (IF) analysis of tissue sections: At necropsy, colons were flushed of content, “Swiss rolled”, and fixed in 10% neutral buffered formalin. Intestinal pathology was assessed via H&E, Sirius Red, and Masson Trichrome staining of colon swiss rolls. Quantification and localization of activated fibroblasts and myofibroblasts was done using IF for smooth muscle actin (SMA) with the Translational Pathology Core (TPL) using Aperio software.
**Bacterial Plating:** Luminal (stool) and mucosally adherent (tissue) bacteria were quantified by serial dilution plating to evaluate colonization and determine bacterial load.

**Molecular Analysis:** Tissue biopsies were analyzed by qPCR for fibroblast activation using the qPCR procedure outlined below.

**Bacterial strains and growth conditions for in vitro assays:** The bacterial strains used in this study are listed in Table 1. All strains were created by the Arthur lab using the lambda-red recombinase method. Bacteria were grown aerobically overnight in shaker at 37°C for all experiments. A bacterial inoculum of MOI 100 was prepared: An aliquot was taken from the culture for optical density (OD600) quantification by spectrometry or quantitative culture. The cultures were then centrifuged thoroughly, the cells were pelleted, and the remaining broth phase was removed by aspiration. The aggregate was re-suspended in phosphate-buffered saline (PBS). A 1:10 dilution in PBS was then taken for quantitative culture or spectrophotometry as a measure of the bacteria concentration of the whole culture. Concentration was calculated using the following formula:

\[ \frac{\text{OD}(600) \times A}{5 \times 10^8 \text{ bacteria/mL}} \]

**Cell Culture:** Swiss 3T3 cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin) at 37°C in a tissue culture incubator. Once confluent (~80%), cells were passaged using Trypsin/EDTA 0.05% and 3.0 x 10^5 cells/mL were split into a new T75 flask.

**Bacteria stimulation experiments:** Six-well plates containing growth media (2 mL) were seeded with Swiss 3T3 cells at a concentration of 3.0 x 10^5 cells/well (~1 mL cells) and incubated at 37°C for 24 hours. Cells were then serum starved by replacing growth media with an equal volume of DMEM.
supplemented with 0.1% heat inactivated FBS and incubated at 37°C for 24 hours. Cells were stimulated with *E. coli* strains (Table 1) at MOI 100 for 4, 24, or 48 hours. Bacteria were killed after 4 hours by aspirating media and replacing with an equal volume of recovery media (serum starve media supplemented with 200 ng/μL gentamycin). PBS was added to cells to serve as a negative control. Cells were stimulated with TGF-β (10 ng/μL) or TGF-β (50 ng/μL) to serve as a positive control. Each stimulant was plated in duplicate or triplicate for all experiments. After collecting cell supernatants (1 mL), Swiss 3T3s were harvested in Trizol (1 mL) for RNA extraction. See Figure 2.

**Figure 2. Experimental design for in vitro fibroblast stimulations with bacteria.**

**Siderophore stimulation experiments:** Six-well plates were seeded with 3.0 x 10⁵ Swiss 3T3 cells/well into growth media (2 mL) and incubated at 37°C for 24 hours. Cells were serum starved like above and incubated at 37°C for 24 hours. Cells were stimulated as listed in Table 2 for 1, 4, 24, or 48 hours. Each stimulant was assayed in duplicate or triplicate for all experiments. After collecting cell supernatants (1 mL), Swiss 3T3s were harvested in Trizol (1 mL). See Figure 3.
**RNA isolation and real-time qPCR analysis:** RNA was extracted with Trizol according to manufacturer’s protocol. The purity of the RNA was evaluated by spectrophotometry using NanoDrop ND-1000 (Thermo Fisher) at a 260:280 and 260:230 ratio. cDNA was synthesized for real-time PCR (RT-qPCR) analysis. Total RNA (500 ng) was reverse transcribed using the qScript cDNA SuperMix (Quantabio). Quantitative real-time PCR was conducted using Sensifast SYBR No-ROX Kit (Bioline) on the Quant Studio 6 (Applied Systems) machine with the following conditions: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, 58°C for 15 seconds, and 72°C for 15 seconds. The expression of several fibrosis-associated genes (Table 3) was analyzed. The genes selected were known to be implicated in fibrotic processes. RT-qPCR SYBR green chemistry was used to quantitate the relative gene expression levels of the fibrotic factors: collagen-1α1, collagen-1α2, collagen-4α1, collagen-6α1, fibronectin, TGF-β1, TGF-β type II receptor, Slit3, and MMP-9. Relative expression was measured relative to *gapdh* housekeeping gene using the ΔΔCt method.

**Data analysis:** *In vivo* experiments were performed reproducibly using two individual cohorts. For *in vitro* experiments, multiple independent biological replicates were performed on separate days, with
each sample being plated in triplicate each day (or duplicate due to lack of materials). Each gene was assayed in triplicate by RT-qPCR. Gene expression was normalized to gapdh and fold expression over unstimulated 3T3 fibroblasts was determined via the $\Delta\Delta$Ct method. The groups were compared using parametric tests only for normally distributed data, and multiple-comparison correction was used where appropriate (Graphpad Prism software). A p-value of less than 0.05 was considered significant.

**Results**

**Histopathological evidence of fibrosis occurred in a subset of NC101-colonized Il10$^{-/-}$ mice.**

While manipulation of the Ybt/FyuA system had a minimal impact on inflammation, a subset of $\Delta$fyuA-colonized Il10$^{-/-}$ mice underwent distinct colonic remodeling, which was observed in haematoxylin and eosin (H&E) stained colon sections at 10 weeks (Fig. 4). The pathology was characterized by an expansion of the submucosa and deposition of wispy, fibrillar substances (Fig. 4D). Because collagen is a characteristic feature of fibrotic tissues, we determined if collagen was present in regions of ECM deposition. Positive staining of the area by both Masson’s trichrome (Fig. 4F) and Sirius red (Fig. 4E) determined it was collagenous in nature. While the collagen matrix in the NC101 colons was structured and organized (Fig. 4B), the collagen in the $\Delta$fyuA colons was expanded and disorganized (Fig. 4E). To summarize, a subset of AIEC-colonized Il10$^{-/-}$ mice developed histopathological lesions consistent with fibrosis.
Figure 4. Deletion of *fyuA* in NC101 *E. coli* promotes inflammation-associated fibrosis in *Il10*⁻/⁻ mice.

Colonization with *fyuA*-deficient *E. coli* NC101 promotes inflammation-associated fibrosis that is associated with a distinct pro-fibrotic colonic transcriptional gene signature. Fibrosis was most severe in *ΔfyuA*-colonized mice (Fig. 5A-B). Since variations in fibrosis severity could be caused by different host responses to the different bacterial strains, we conducted high-throughput RNA sequencing to determine whether global differences were apparent in the colonic transcriptomes of inflamed *Il10*⁻/⁻ and non-flamed WT mice colonized with NC101 or *ΔfyuA* (pro-fibrotic strain). Principal Coordinate Analysis (PCoA) revealed significant differences in the colonic transcriptomes of NC101 versus *ΔfyuA*-colonized *Il10*⁻/⁻ mice at 10 weeks. Gene-level and pathway analysis revealed 2692 genes that were differentially expressed between *ΔfyuA*-colonized fibrotic *Il10*⁻/⁻ mice versus NC101-colonized *Il10*⁻/⁻ mice at 10 weeks (Fig. 6). The most highly upregulated gene ontology pathway was extracellular matrix with a magnitude of perturbation of 7.4 (Fig. 6). Differential expression of this pathway was not evident in WT mice, nor was fibrosis. Together these results demonstrate we have developed a new animal model for IBD-associated fibrosis, with fibrosis confirmed via histologic and molecular methods.
Figure 5. Yersiniabactin biosynthesis promotes fibrosis in AIEC-driven colitis.

<table>
<thead>
<tr>
<th>NC101 strain</th>
<th>Fibrosis incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>30.4%</td>
</tr>
<tr>
<td>ΔfyuA</td>
<td>58.6%</td>
</tr>
<tr>
<td>Δirp1</td>
<td>18.2%</td>
</tr>
<tr>
<td>ΔfyuA Δirp1</td>
<td>6.7%</td>
</tr>
</tbody>
</table>

Fig 5. Fibrosis incidence rates in Il10−/− mice monoassociated with NC, ΔfyuA, Δirp1, or ΔfyuA Δirp1 for 10 weeks as assessed by H&E histology (a-c). The Ybt-positive group includes Il10−/− mice colonized with NC or ΔfyuA. The Ybt-negative group includes Il10−/− mice colonized with Δirp1 or ΔfyuA Δirp1. Composite fibrosis histology scores at 10 weeks (d). Each symbol represents an individual mouse (n = 11-29).

Figure 6. Colon tissue with fibrotic pathology had a significantly different host gene signature as assessed by RNAseq.

Fig 6. RNAseq was performed on host RNA isolated from proximal colon tissue. Principle coordinate analysis reveals transcriptome-wide changes in the colons of E. coli NC101 vs. NC101ΔfyuA colonized WT and Il10−/− mice 10 weeks post-colonization. The table shows the top five upregulated pathways in host transcriptome. Each symbol represents an individual mouse.
Yersiniabactin biosynthesis is necessary for induction of inflammation-associated fibrosis.

The FyuA receptor is responsible for the import of Ybt-metal complexes as part of the Ybt siderophore system. We hypothesized that disruption of Ybt uptake contributed to the pro-fibrotic potential of NC101ΔfyuA, and that colonization with a Ybt-deficient strain, NC101Δirp1, would also induce fibrosis. To determine if inactivation of Ybt biosynthesis would also promote induction of fibrosis, the Δirp1 mutant was generated which prevents the biosynthesis of Ybt. Surprisingly, only 18.2% of Δirp1 mice developed histological evidence of fibrosis compared to 58.6% of ΔfyuA-colonized mice (Fig. 5a). Next, the ΔfyuA Δirp1 mutant was generated to confirm that Ybt biosynthesis was required for the development of fibrosis. The ΔfyuA Δirp1 mutant also decreased the incidence of fibrosis with only 6.7% of mice showing evidence of fibrosis (Fig. 5a). Taken together (Fig. 5), these data demonstrate that Ybt biosynthesis is required for pro-fibrotic activity, but independent of its uptake through FyuA.

Live E. coli and its Ybt-FyuA uptake system do not stimulate fibroblast activation in vitro.

Because RNAseq analysis revealed the ECM pathways to be the most differentially regulated gene ontogeny category (Fig. 6), we next tested whether bacteria could directly and differentially activate fibroblasts. We developed an in vitro co-culture model of Swiss 3T3 fibroblasts and NC101 E. coli followed by qPCR analysis of pro-fibrotic genes expressed during fibroblast activation. We performed extensive optimization of this in vitro assay before evaluating the impact of the E. coli Ybt/FyuA system on fibroblasts (optimization data not shown). Stimulation at 4, 24, and 48-hour time-points (Fig. 7A-C) revealed the E. coli strains were incapable of directly activating fibroblasts when compared to the positive control TGF-β (10ng/μL). From this, we concluded that live E. coli are unlikely to directly activate fibroblasts in our inflammation-associated colitis model.
Figure 7. Live NC101 *E. coli* did not directly or differentially activate Swiss 3T3 fibroblasts *in vitro*.
B

24 Hour Stimulation

Col1a2

Fold Change to gapdh

PBS  WT  ΔfyuA  ΔfyrΔfyr  ΔfyrAΔfyr  TGF-β (10ng/μL)  TGF-β (50ng/μL)

Col4a1

Fold Change to gapdh

PBS  WT  ΔfyuA

Fn1

Fold Change to gapdh

PBS  WT  ΔfyuA  ΔfyrΔfyr  ΔfyrAΔfyr  TGF-β (10ng/μL)  TGF-β (50ng/μL)

Tgfb1

Fold Change to gapdh

PBS  WT  ΔfyuA  ΔfyrΔfyr  ΔfyrAΔfyr  TGF-β (10ng/μL)  TGF-β (50ng/μL)

Tgfbr2

Fold Change to gapdh

PBS  WT  ΔfyuA  ΔfyrΔfyr  ΔfyrAΔfyr  TGF-β (10ng/μL)  TGF-β (50ng/μL)

Slit3

Fold Change to gapdh

PBS  WT  ΔfyuA
Fig 7. Swiss 3T3 fibroblasts were stimulated with the indicated FyuA/Ybt variant *E. coli* NC101 strains at MOI 100. Extracellular bacteria were killed off at 4 hours with Gentamycin. Cells were harvested after 4 (A), 24 (B), or 48 (C) hours and collected in Trizol to extract RNA. Relative gene expression was quantified via RT-qPCR utilizing SYBR green chemistry. Genes were normalized to the housekeeping gene *Gapdh* using ∆∆Ct method. Each symbol represents a biological replicate (independent experiment) plated in triplicate (within each independent experiment). Lines are at the mean.
Fibroblasts are unlikely to be directly stimulated by bacteria or bacterial products. Because we found live *E. coli* incapable of directly activating fibroblasts, we next examined whether bacterial products that enter intestinal tissues during inflammation were capable of fibroblast activation. This seemed a plausible next step because, in contrast to intestinal epithelial cells and macrophages, intestinal fibroblasts rarely encounter live bacteria, but could encounter their bacterial products. Specifically, we chose to evaluate the effects of the Ybt siderophore and the chelators TPEN, DTPA, and BPD (Table 2).

To do this, we developed an *in vitro* co-culture model of Swiss 3T3 fibroblasts and bacterial products followed by qPCR analysis of pro-fibrotic genes expressed during fibroblast activation. We performed extensive optimization of this *in vitro* assay before evaluating the impact of the siderophore and chelators on fibroblasts (optimization data not shown).

Gene expression of *col4a1* and *Fn1* was increased by DTPA after 4 hours (Fig. 8B, red symbols). It is possible that DTPA also increased the expression of collagens and fibronectin at 24 and 48 hours (Fig. 8C-D, green symbols), but additional experiments would be required to determine if this conclusion is statistically valid. At 24 and 48 hours, both TPEN and Ybt stimulated fibroblasts showed visible evidence of cellular death at harvest, which was further substantiated by low gene expression values (Fig. 8C-D, blue symbols). TPEN and DTPA are both metal chelators with differing specificities for biologically relevant metals; TPEN has a high binding specificity for Zn$^{2+}$, while DTPA is more promiscuous and binds Fe$^{3+}$, Cu$^{2+}$, Ga$^{2+}$, and more (TPEN, DTPA). The Arthur lab is further investigating how zinc depletion with TPEN can induce death of various cell types.
Figure 8. Bacterial products showed minimal activation of fibroblasts.
Figure 8. Swiss 3T3 fibroblasts were stimulated with Ybt and various chelators in the concentrations indicated. Cells were harvested after 1 (A), 4 (B), 24 (C), or 48 (D) hours and collected in Trizol for RNA extraction. Relative gene expression was quantified via RT-qPCR utilizing Sybr green chemistry. Genes were normalized to the housekeeping gene Gapdh using ∆∆Ct method. Each symbol represents a biological replicate plated in triplicate. Lines are at the mean. Red symbols indicate a statistical difference calculated via t-test (p<0.05). Blue symbols indicate evidence of substantial cell death at harvest. Green symbols indicate stimulations of interest that require further experimental replicates.
**Discussion**

Fibrosis afflicts more than 30% of patients with IBD, yet there is no cure and few, inadequate treatment options. One major reason is due to lack of animal models. We have developed an inflammation-associated fibrosis animal model, which will permit further research and lead to the development of new treatment options.

Recent studies have implicated the importance of perturbations of the gut microbiota in the onset of IBD. Now, we have linked a pathobiont of the gut microbiota (*E. coli* NC101) to the development of IBD-associated fibrosis. Moreover, our findings substantiate that we may have linked a bacterial small molecule (Ybt) from the gut microbiota to fibrosis. As many gut bacteria are capable of producing siderophores and metallophores, such findings could be extended to multiple microbial-associated impacts on human health and disease.

*In vivo* studies revealed a subset of *Il10*−/− mice monoassociated with NC101 ΔfyuA developed a fibrotic pathology. This indicates Ybt may favor the development of fibrotic IBD over inflammatory IBD. The attenuation of fibrotic development by the Δirp1 mutant demonstrated the fibrotic phenotype was not driven by the inability to import Ybt. Further abrogation of the pathology by the ΔfyuA Δirp1 mutant indicated Ybt biosynthesis was required to induce fibrosis. This, along with no evidence of differences in host or bacterial iron homeostasis, suggests a novel mechanism for the Ybt siderophore system in addition to its role in iron chelation. Thus, we have demonstrated uncoupling of biosynthesis and uptake of the Ybt siderophore.
In vitro studies revealed that neither WT NC101 E. coli nor its isogenic mutants were capable of directly activating fibroblasts. While these experiments failed to identify a cell-type targeted by bacteria, they prompted us to investigate the impact of bacterial products on fibroblast activation.

Stimulation of fibroblasts with the chelator DTPA demonstrated a potential increase in the expression of collagen and fibronectin, which are hallmark genes of fibrosis and formation of the ECM. This suggests that the in vivo disease phenotype might function mechanistically similar to DTPA metal chelation. DTPA is a promiscuous chelator capable of binding iron, copper, nickel, zinc, and others. Ybt can also bind these biologically important metals at varying affinities.

In vitro stimulations repeatedly showed that excessive Zn\(^{2+}\) chelation via TPEN caused fibroblast cell death. Stimulation with Ybt (5\(\mu\)M) showed a similar gene expression profile to TPEN at 24 and 48 hours, which are the same time-points that show evidence of TPEN-induced cell death. Taken together, this suggests that excessive Zn\(^{2+}\) chelation causes the death of fibroblasts. The Arthur lab is currently performing additional studies to titrate Ybt and chelators in fibroblasts, intestinal epithelial cells, and macrophages.

At present, the impact of bacterial products on fibroblasts remains unclear. However, our findings have provided considerable evidence for ruling out the direct stimulation of fibroblasts as part of the mechanism involved in fibrogenesis. We therefore have decided to focus future studies on other cell types that could be impacting fibroblast activation, such as macrophages. Current and future studies will evaluate how the NC101 Ybt-FyuA system impacts macrophage function, including M1/M2 skewing and the pro-fibrotic behavior of macrophages.
### Tables

**Table 1. Bacterial strains used in this study.**

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Sufficient in Ybt</th>
<th>Sufficient in FyuA</th>
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<tbody>
<tr>
<td>NC101 WT</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>NC101 ΔfyuA</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>NC101 Δirp1</td>
<td>no</td>
<td>yes</td>
</tr>
<tr>
<td>NC101 ΔfyuA Δirp1</td>
<td>no</td>
<td>no</td>
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**Table 2. Bacterial small molecules used in this study.**

<table>
<thead>
<tr>
<th>Small Molecule</th>
<th>Mechanism</th>
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<tbody>
<tr>
<td>Diethylenetriaminepentaacetic acid (DTPA)</td>
<td>Promiscuous chelator</td>
</tr>
<tr>
<td>N,N',N″-Tetrakis(2-pyridinylmethyl)-1,2-ethanediamine (TPEN)</td>
<td>Zinc chelator</td>
</tr>
<tr>
<td>2,2'-bipyridyl (BPD)</td>
<td>Iron chelator</td>
</tr>
<tr>
<td>Yersiniabactin (Ybt)</td>
<td>Experimental siderophore</td>
</tr>
<tr>
<td>Transforming growth factor 1 (TGF-β)</td>
<td>Master regulator of fibrosis</td>
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**Table 3. Oligonucleotide primers used in this study.**

<table>
<thead>
<tr>
<th>Name</th>
<th>Forward Primer (5’ to 3’)</th>
<th>Reverse Primer (5’ to 3’)</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>gapdh</td>
<td>GGTGAAGGTCGAGTGCAACGGA</td>
<td>GAGGGATCTCGCTCTGGGAAGA</td>
<td>housekeeping gene</td>
</tr>
<tr>
<td>mCol1a1</td>
<td>TGACTGGAAGAGCGGAGAGT</td>
<td>ATCCATCGGTCATGCTCTCT</td>
<td>type I collagen</td>
</tr>
<tr>
<td>mCol1a2</td>
<td>AGGCCCACCTGTAACACC</td>
<td>CTGAGAAGCAGGTTGGCTA</td>
<td>type II collagen</td>
</tr>
<tr>
<td>mCol4a1</td>
<td>CACAGTCAGACCATTGCAGATTC</td>
<td>CGGAACCTTCAGACCCAG</td>
<td>type IV collagen</td>
</tr>
<tr>
<td>mCol6a1</td>
<td>CTGCTGGTGAGATGGATGGGTG</td>
<td>TGTGCGAGTCATAGCCGAATAG</td>
<td>type VI collagen</td>
</tr>
<tr>
<td>mFn1_</td>
<td>CCAAACCTCTTGTGTGTTCA</td>
<td>GAGAGCTTCTGTCTCTCT</td>
<td>fibronectin (ECM component)</td>
</tr>
<tr>
<td>mTgfb1_</td>
<td>TCTCTGTGGAGCTGAAGCAA</td>
<td>TGAGTGGCTGTCTTTTGACG</td>
<td>master regulator of fibrosis</td>
</tr>
<tr>
<td>mTgfbr2</td>
<td>AGCATCAGGCCATCTGTG</td>
<td>TGGCAAACCGTCTCCAGAGT</td>
<td>TGF-β receptor</td>
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<tr>
<td>Slit3</td>
<td>GCTTGTGCACCATTGGCCC</td>
<td>CTCTCCAGCTGCTTCAGATC</td>
<td>3T3 activation gene</td>
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<tr>
<td>mMmp-9</td>
<td>CCTTGGTGAGCACAACAGC</td>
<td>ATACTGGATGCGCTCATCTGCG</td>
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</table>

*These genes have been identified as being differentially regulated in vivo by the Arthur Lab’s RNA-seq studies and are known to influence fibrosis.*
Acknowledgements:
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References


