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UNIVERSITY OF CALIFORNIA SAN DIEGO

Remodelling tumor microenvironments through host-microbial interfaces

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Bioengineering

by

Katherine O'Connor

Committee in charge:

Professor Jeff Hasty, Chair
Professor Adam Engler
Professor Esther Kwon
Professor Christian Metallo

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The Dissertation of Katherine O'Connor is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

University of California San Diego

2026

DEDICATION

To my acquired family, for leading by example, sparking curiosity, and providing security to embrace change.

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VITA

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ABSTRACT OF THE DISSERTATION

Remodelling tumor microenvironments through host-microbial interfaces

by

Katherine O'Connor

Doctor of Philosophy in Bioengineering

University of California San Diego, 2026

Professor Jeff Hasty, Chair

Colorectal cancer is characterized by a fibrotic phenotype that makes solid tumors hard to treat. The overexpressed and dysregulated extracellular matrix limits the delivery of therapeutics and the infiltration of immune cells, presenting a physical barrier that inhibits or dampens therapeutic effects and full tumor perfusion. Here we show that introducing quorum-regulated collagenase expression into attenuated *Salmonella typhimurium* enables bacteria to remodel this barrier from within the tumor and improve its own colonization efficiency. We demonstrate that collagenase expression under LuxI/LuxR quorum-sensing control produces density-dependent ECM disruption in CRC spheroids and significantly enhances intratumoral bacterial penetration at physiologically relevant seeding densities. We establish a patient-derived

organoid co-culture platform using matched tumor and normal colon tissue from microsatellite-stable and microsatellite-unstable CRC patients, and show that *Salmonella* achieves tumor-selective colonization that is coupled to quantifiable morphological and transcriptional responses. When the collagenase strain is co-administered in consortia (i.e. with a therapeutic proxy strain), it significantly enhances partner colonization in patient-derived CRC organoids from both molecular subtypes. A pilot in vivo study in the MC38 syngeneic model shows directional trends toward reduced intratumoral collagen and improved immune cell penetration depth. These findings support a therapy primer strategy in which an ECM-remodeling bacterial strain sensitizes the tumor microenvironment to amplify the efficacy of existing therapeutic modalities without requiring new agents.

Introduction

0.1 Cancer remains a disease of the tumor microenvironment

Colorectal carcinoma is among the most common and lethal malignancies worldwide [41]. Despite meaningful advances in surgery, chemotherapy, and immunotherapy, outcomes in advanced disease remain poor, and a significant fraction of treatment failures occur not because drugs lack intrinsic activity but because they fail to reach tumor cells in sufficient concentration [30, 40]. Solid tumors are not homogeneous masses of malignant cells. They are structured tissue compartments that include stromal fibroblasts, immune populations, vasculature, and an extensive extracellular matrix, each of which shapes drug penetration, immune access, and the selective pressures that drive tumor evolution [3, 34].

The extracellular matrix poses a particular barrier to therapy in CRC. Colorectal tumors are frequently desmoplastic, characterized by deposition of fibrillar collagen by cancer-associated fibroblasts and dysregulated matrix metalloproteinase activity that shifts the balance toward net collagen accumulation [45, 53]. The resulting dense, stiffened matrix impedes diffusion of small molecules and macromolecules into the tumor parenchyma, compresses intratumoral vasculature and exacerbates hypoxia, and physically restricts immune cell migration [52, 24, 35]. Tumor hypoxia is itself a driver of therapeutic resistance: hypoxic regions are refractory to ionizing radiation [26], poorly served by chemotherapeutics that depend on active vascular delivery or cell cycling [54], and transcriptionally rewired through HIF-1 α toward metabolic reprogramming and invasive behavior [50, 17, 56, 18]. The tumor core is therefore both the most treatment-resistant

compartment and the least accessible one.

Immunotherapy has partially addressed delivery barriers by mobilizing endogenous effectors, but its success in CRC is largely restricted to microsatellite-unstable disease, which accounts for roughly 15% of metastatic CRC [43]. The majority of CRC is microsatellite-stable, with an immune-excluded microenvironment in which T cells are present in the surrounding stroma but fail to penetrate the tumor [3, 60]. Collagen is mechanistically implicated in this exclusion, providing both physical impediment to T cell migration and matrix-derived signals that suppress effector function [1]. Improving outcomes in MSS CRC requires changing the physical and immune composition of the tumor, not just altering signaling.

0.2 Bacteria as tumor-colonizing therapeutic agents

The observation that microorganisms can interact with tumors therapeutically has a long history. Coley's toxins, preparations of killed bacteria administered to cancer patients in the 1890s, produced durable responses in some cases and established an early precedent for microbial involvement in antitumor immunity [7]. Intravesical BCG, introduced in the 1970s, remains the standard of care for non-muscle-invasive bladder cancer and represents the longest continuously used bacterial cancer therapy [37].

The modern rationale for using live bacteria in solid tumors rests on the observation that certain facultative and obligate anaerobes colonize tumor cores preferentially after systemic administration [58, 59]. This tropism reflects a convergence of ecological factors: immune-privileged hypoxic and necrotic regions provide shelter from phagocytic clearance, necrotic tissue supplies nutrients that support bacterial growth, and the low oxygen tension and acidic pH of the tumor core select for organisms capable of anaerobic or microaerophilic metabolism [21, 13]. Attenuated *Salmonella typhimurium* has been shown to accumulate in murine tumor models at densities orders of magnitude higher than in normal tissue after intravenous administration, and has been evaluated in patients with advanced solid tumors in early-phase trials [55, 39].

Synthetic biology has substantially expanded what can be done with this tumor tropism. Rather than relying solely on the immunostimulatory properties of bacteria, engineered strains can be programmed with gene circuits that sense TME-specific cues and respond by producing defined payloads at controlled times and locations within the tumor [5, 27, 46, 29]. Strains have now been engineered to deliver checkpoint blockade nanobodies locally [28], recruit and coordinate immune responses through chemokine expression [49], encode neoantigens for personalized immunotherapy [47], detect tumor-specific DNA [8], and improve colonization specificity through biosensing of tumor metabolites [6]. In one notable recent example, bacteria expressing tumor-localized antigens were used to guide CAR-T cells to solid tumors, positioning bacteria as a delivery infrastructure for an existing cellular therapy rather than as an independent therapeutic agent [57]. The broader field has been reviewed comprehensively elsewhere [19, 2].

A consistent observation across this body of work is that bacteria, by virtue of their tumor tropism and proliferative capacity, function as self-amplifying delivery vehicles that reach compartments inaccessible to most conventional agents. The challenge is to add therapeutic precision and maintain biosafety, and to do so without compromising the colonization fitness that makes the approach viable.

0.3 Quorum sensing and the synchronized lysis circuit

One approach to controlled payload delivery in bacteria is the use of quorum-sensing circuits, in which gene expression is coupled to population density through the accumulation of small diffusible autoinducer molecules. In gram-negative bacteria, LuxI/LuxR-based systems produce and sense N-acyl homoserine lactones (AHL) that accumulate as a function of local bacterial concentration; when AHL crosses a threshold, it complexes with LuxR to activate downstream transcription. This density-dependent gating provides an intrinsic form of spatial selectivity: expression is activated only where bacteria accumulate to sufficient density, which in the context of tumor therapy corresponds to the colonized tumor core rather than peripheral or

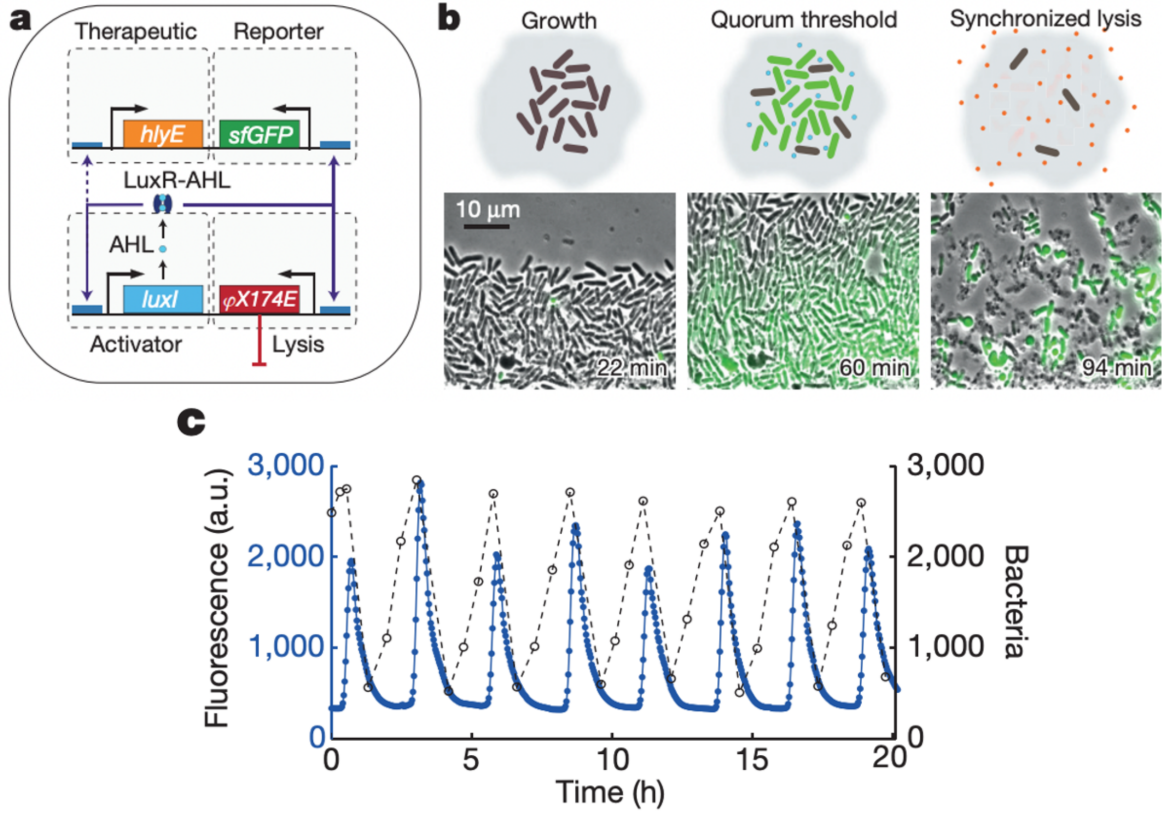


Figure 1. The synchronized lysis circuit (SLC) couples quorum sensing to payload delivery and population-level lysis. (A) Schematic of the SLC gene circuit architecture. The activator module encodes *luxI*, which produces the AHL autoinducer; AHL accumulates as a function of population density and, upon reaching threshold, complexes with LuxR to activate transcription of the therapeutic payload (*hlyE*), the GFP reporter (*sfGFP*), and the lysis gene (ϕ X174E). The lysis module drives synchronized population collapse, resetting AHL below threshold and initiating the next growth cycle. (B) Schematic (top) and representative fluorescence micrographs (bottom) of the three stages of SLC behavior in microfluidic culture: growth (22 min), AHL accumulation and GFP activation at quorum threshold (60 min), and synchronized lysis (94 min). Scale bar, 10 μ m. (C) Representative time-series trace of SLC oscillatory dynamics over 20 hours. Blue solid line with filled circles: GFP fluorescence (a.u., left axis). Black dashed line with open circles: bacterial cell count (right axis). Periodic peaks in fluorescence coincide with population density peaks, followed by sharp drops corresponding to synchronized lysis events. Adapted from Din et al., 2016 [12].

normal tissue.

The synchronized lysis circuit (SLC), developed in the Hasty laboratory, uses this architecture to drive oscillatory population dynamics [12]. Bacteria grow to quorum, activate expression of both a therapeutic payload (*hlyE*) and a lysis gene (ϕ X174E), undergo synchronized population collapse, and then regrow from surviving cells to repeat the cycle. This produces periodic pulses of payload release that are autonomous and require no exogenous induction [1]. The circuit has been validated for in vivo payload delivery across multiple tumor models [12, 6], and its modular architecture makes it straightforward to swap the payload while preserving the regulatory structure.

This modularity is the basis for the collagenase construct described in Aim 1. The *hlyE* therapeutic cassette is replaced with a codon-optimized gene encoding the membrane-anchored *Streptomyces omiyaensis* trypsin (SOT) collagenase, and the lysis module is inactivated to generate a non-lytic, sustained membrane-display variant. The decision to use quorum regulation rather than constitutive or inducible expression is not simply a matter of circuit aesthetics. Bacterial collagenases are recognized virulence factors whose constitutive expression in a tumor-colonizing organism would create potential for off-target matrix degradation at any site of bacterial colonization, including non-tumor tissue [38]. The quorum threshold makes enzyme expression contingent on the high local density characteristic of established intratumoral colonization, using the same ecological selectivity of the chassis to gate the collagenolytic activity of the displayed enzyme.

0.4 ECM remodeling as a bacterial therapeutic strategy

Several groups have explored bacterially delivered ECM-degrading enzymes as a way to improve intratumoral penetration. Ebelt et al. showed that constitutive collagenase expression in *Salmonella* reduces intratumoral collagen in pancreatic ductal adenocarcinoma models, decreases immunosuppressive cell populations, and reduces tumor growth [15]. A

companion study demonstrated similar results with hyaluronidase-expressing *Salmonella* targeting tumor-associated hyaluronic acid [16]. Li and Ye have since described a probiotic-based collagenase delivery system with evidence of tumor penetration enhancement [38]. Collagenase administered intraperitoneally has also shown activity in a murine model of colorectal peritoneal carcinomatosis [22]. The mechanistic basis for these effects is consistent with the known role of tumor-associated collagen in restricting drug and immune cell access to the tumor parenchyma [53, 33, 52].

These studies establish feasibility but share a design limitation: inducible or constitutive expression systems provide limited spatial control, and the biosafety concerns associated with constitutive collagenase expression in a systemically administered bacterium have not been systematically addressed. The present work approaches ECM remodeling with a different framing. Rather than asking whether bacterially delivered collagenase can reduce tumor burden on its own, we ask whether it can lower the colonization threshold for a co-administered therapeutic partner, functioning as what we refer to as a therapy primer. This sets a different efficacy bar: the collagenase strain does not need to independently drive tumor regression, but rather condition the microenvironment in a way that improves the performance of another intervention. The primer concept connects naturally to existing approved therapies (i.e. chemotherapy, checkpoint inhibitors, cellular therapies) all of which face the same ECM penetration problem that collagenase is designed to address [52, 19].

0.5 Patient-derived organoids as preclinical models

Most preclinical work on bacterial cancer therapeutics has used immortalized cell lines that have been adapted to culture conditions for decades and that differ substantially from primary tumors in mutational landscape, ECM composition, and microenvironmental dependencies [14]. Three-dimensional tumor spheroids generated from these lines better recapitulate the hypoxic and necrotic gradients that support bacterial colonization [31], but they still lack

patient-specific biology and the ability to make tumor-versus-normal comparisons in the same genetic background.

Patient-derived organoids address both limitations. Established directly from primary surgical resection specimens, they preserve the epithelial architecture, somatic mutational context, and growth factor dependencies of the original tumor. Matched normal colon organoids from the same patient provide an internal control for tumor specificity that monoculture models cannot. CRC PDOs have been validated as predictive models for patient drug response across multiple therapeutic classes, making them a more clinically interpretable test bed for bacterial therapeutic candidates than standard cell line spheroids [29].

The computational morphometric pipeline developed in Aim 2 of this dissertation adds analytical utility to this system. By demonstrating that brightfield features (particularly integrated density) can classify high bacterial colonization state with $AUC = 0.971$ without any fluorescent reporter, the pipeline enables evaluation of untagged strains that cannot carry GFP for biosafety or metabolic reasons. This is a practical consideration for moving toward clinical-stage candidates, where reporter transgenes are not acceptable, and it is directly applied in Aim 3 to evaluate the untagged ST-pLux-Collagenase strain.

0.6 Scope of this dissertation

This dissertation asks whether quorum-regulated collagenase expression in engineered *Salmonella typhimurium* can reduce the ECM barrier to bacterial colonization in colorectal cancer models and, in doing so, improve the colonization efficiency of a co-administered therapeutic partner strain. The work proceeds across three experimental systems of increasing biological complexity.

In Chapter 1, we characterize arabinose-inducible and quorum-regulated collagenase expression in HCT-116 spheroids, demonstrate density-dependent ECM disruption by the quorum-regulated strain, and show that it significantly improves intratumoral bacterial penetration relative

to a parental control at low physiologically relevant seeding density.

In Chapter 2, we establish a patient-derived organoid co-culture platform using matched tumor and normal colon tissue from MSS and MSI CRC patients, characterize the colonization performance of *S. typhimurium* and *E. coli* Nissle 1917 across multi-modal readouts, develop and validate a brightfield morphometric pipeline as a fluorescence-independent colonization classifier, and analyze cross-patient conservation of morphometric and transcriptional responses.

In Chapter 3, we evaluate the ST-pLux-Collagenase strain in the PDO system without fluorescent reporters, demonstrate collagenase-specific colonization enhancement and morphometric and transcriptional remodeling in both patient lines, provide direct experimental evidence for the therapy primer concept through a co-inoculation experiment, and present pilot in vivo data from the MC38 syngeneic mouse model showing directional trends consistent with collagenase-mediated ECM remodeling and improved immune infiltration.

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Chapter 1

Quorum-Regulated Collagenase Expression in Engineered *Salmonella typhimurium* for Enhanced Tumor Colonization

1.1 Summary

The dense extracellular matrix (ECM) of solid colorectal tumors constitutes a fundamental physical barrier to therapeutic delivery, limiting the intratumoral penetration of both small-molecule agents and living therapeutics including bacteria. This chapter describes the engineering and functional validation of a collagenase-expressing *Salmonella typhimurium* strain 1301 (ST-pLux-Collagenase) designed to autonomously degrade tumor-associated collagen and thereby enhance its own intratumoral penetration. As a foundation for this work, we first replicated prior proof-of-concept literature demonstrating that arabinose-inducible collagenase expression in *Salmonella* (pBad-Col) is compatible with bacterial viability across a range of inducer concentrations and produces measurable ECM disruption in HCT-116 colorectal cancer spheroids, establishing membrane-anchored collagenase as a feasible payload for this chassis. We then engineered a quorum-sensing-regulated collagenase strain by replacing the therapeutic cassette of the synchronized lysis circuit (SLC) with a codon-optimized gene encoding the *Streptomyces omiyaensis* trypsin (SOT) collagenase fused for outer membrane display, while retaining the quorum-activated expression architecture and deleting the lysis module to generate

a non-lytic, membrane-display variant (ST-pLux-Collagenase). This circuit design ensures that SOT is expressed only upon accumulation of bacteria to sufficient intratumoral density, providing an inherent tumor-specificity safeguard by coupling enzyme production to colonization burden. Characterization of this strain across three bacterial seeding densities in HCT-116 spheroids using the bacteria-spheroid co-culture (BSCC) platform demonstrated density-dependent collagenase activity and ECM disruption, with the most striking phenotype at high seeding density, where spheroid architecture underwent rapid and visually dramatic dissolution. At low seeding density most relevant to the physiological colonization conditions expected in vivo, a GFP-tagged version of the collagenase strain (ST-pLux-Collagenase-GFP) showed significantly superior intratumoral penetration compared to the parental 1301-GFP control at both 24 hours ($**p < 0.01$, $***p < 0.001$) and 5 days ($*p < 0.05$), demonstrating that quorum-regulated SOT expression enhances bacterial colonization efficiency in a three-dimensional tumor model under physiologically relevant conditions.

1.2 Results

1.2.1 The synchronized lysis circuit: a quorum-sensing platform for tumor-localized payload delivery

Effective bacterial cancer therapeutics require not only tumor-selective colonization but also spatiotemporally controlled payload delivery that is coupled to bacterial density within the tumor rather than to exogenous inducers that may have poor intratumoral bioavailability. The synchronized lysis circuit (SLC), originally developed by Din et al. [12], provides a principled solution to this challenge through a LuxI/LuxR quorum-sensing architecture in which bacterial population density is transduced into a transcriptional switch that activates payload expression. In the canonical SLC, the *luxI* autoinducer synthase gene drives accumulation of N-acyl homoserine lactone (AHL) as a function of population density; when AHL reaches a threshold concentration, it complexes with the LuxR receptor protein to activate expression of both the therapeutic payload

(*hlyE*, encoding hemolysin E) and a reporter (*sfGFP*), while simultaneously inducing the phage lysis gene ϕ X174E to cause synchronized bacterial lysis and payload release (Figure 1A). This architecture produces oscillatory population dynamics in which cycles of quorum-triggered lysis reset the population below threshold, followed by regrowth to quorum, generating the characteristic periodic fluorescence and cell count oscillations observed in microfluidic culture (Figure 1B–C).

The SLC represents a powerful engineering chassis for tumor-localized therapeutics because the AHL quorum threshold is intrinsically tied to local bacterial density, meaning payload release is spatially restricted to sites of active colonization and scales with colonization burden. In vivo, this architecture confers an additional layer of tumor selectivity: bacteria that colonize peripheral or normal tissues at low density fail to reach quorum and therefore do not activate therapeutic expression, while bacteria that accumulate in the hypoxic and necrotic cores of solid tumors, where immune clearance is reduced and nutrient gradients favor anaerobic growth, achieve the high local densities required for circuit activation [12, 10]. The work described in this chapter builds directly on the SLC platform by replacing the hemolysin therapeutic cassette with the codon-optimized SOT collagenase gene displayed on the outer bacterial membrane, with the dual goal of remodeling the tumor ECM to improve bacterial penetration and establishing a precedent for circuit-adapted enzymatic integration.

1.2.2 Arabinose-inducible collagenase expression in *Salmonella* confirms ECM-degrading activity and growth compatibility across inducer concentrations

Before engineering a quorum-regulated collagenase circuit, we first sought to establish proof of concept for collagenase expression in *Salmonella typhimurium* 1301 using an inducible system, replicating and adapting the approach of Ebelt et al. [15], who demonstrated arabinose-inducible collagenase activity in a *Salmonella* chassis. A plasmid encoding bacterial collagenase under the control of the arabinose-inducible pBad promoter (pBad-Col) was transformed into

ST 1301, producing the pBad-Col strain (Figure 1.1A). This inducible system allows titration of collagenase expression level through the arabinose concentration supplied to the culture medium, providing a clean experimental handle for characterizing both the metabolic burden of collagenase expression on bacterial growth and the downstream ECM-degrading phenotype in tumor spheroids.

Bacterial growth curves at arabinose concentrations of 0%, 1%, 2.5%, and 5% revealed a dose-dependent suppression of maximum OD₆₀₀ with increasing inducer concentration (Figure 1.1B). At 0% arabinose (uninduced), bacteria reached a maximum OD₆₀₀ of approximately 1.1, consistent with normal stationary-phase growth. At 1% arabinose, maximum OD₆₀₀ was modestly reduced to approximately 0.85, while 2.5% and 5% arabinose produced near-equivalent further suppression to approximately 0.70–0.72. The dose-dependent growth suppression at higher arabinose concentrations is consistent with metabolic burden imposed by high-level collagenase protein production, a well-described phenomenon in recombinant protein expression systems [25]. Importantly, bacterial viability was maintained at all tested arabinose concentrations. Cultures at 5% arabinose, while growing more slowly, still reached substantial OD values and did not exhibit aberrant lysis or growth arrest, indicating that pBad-driven collagenase expression at these levels is compatible with bacterial fitness.

To assess whether arabinose-induced collagenase expression translates to ECM-degrading activity in a three-dimensional tumor context, we co-cultured pBad-Col bacteria with HCT-116 colorectal carcinoma spheroids under the BSCC protocol [31], comparing uninduced (0% arabinose, top row) and induced (5% arabinose, bottom row) conditions at 0, 24, and 68 hours post-inoculation. Representative brightfield images demonstrate a striking and progressive difference between conditions (Figure 1.1C). Uninduced spheroids maintained their dense, well-defined architecture across the full 68-hour imaging window, with a compact core and smooth peripheral boundary consistent with normal tumor spheroid morphology. In contrast, induced spheroids showed progressive dissolution of the ECM scaffold beginning at 24 hours, with visible disruption of the peripheral fibrous matrix and loss of structural definition, advancing to marked

darkening and compaction of the spheroid core by 68 hours. This phenotype is consistent with collagenase-mediated degradation of the collagen-rich pericellular matrix that maintains spheroid three-dimensional integrity, analogous to results reported in the Ebel et al. study and consistent with the known role of matrix metalloproteinases and bacterial collagenases in disrupting tumor ECM architecture [15, 52].

Two accompanying time-lapse videos (Supplementary Video 1, uninduced; Supplementary Video 2, induced) provide real-time visualization of the differential spheroid response to arabinose-induced collagenase expression. These videos capture the progressive, temporally continuous nature of the ECM disruption phenotype and complement the static brightfield stills in Figure 1.1C. Notably, the induced condition shows bacteria-driven dissolution of the outer spheroid matrix that proceeds inward over time, consistent with the expected mode of action of a membrane-anchored bacterial collagenase that is displayed on the outer bacterial membrane and acts locally on contact with the surrounding collagen matrix.

Together, these data establish that (i) pBad-driven collagenase expression in ST 1301 is metabolically tolerated at all tested arabinose concentrations, (ii) higher inducer concentrations impose a quantifiable but non-lethal metabolic burden, and (iii) induced collagenase activity produces functional ECM disruption in HCT-116 spheroids over a 68-hour co-culture window. These findings validate the feasibility of membrane-anchored SOT collagenase expression as a functional ECM-degrading modality for *Salmonella*-based tumor therapy and motivated the engineering of a quorum-regulated delivery system to replace the constitutive inducer requirement.

1.2.3 Integrating membrane-anchored SOT into the SLC architecture

Having established collagenase expression compatibility in the ST 1301 chassis, we next engineered a quorum-regulated collagenase strain by adapting the SLC architecture. The canonical SLC circuit encodes four functional modules: a therapeutic payload module (originally *hlyE*), a GFP reporter module (*sfGFP*), an activator module (*luxI*), and a lysis module (ϕ X174E;

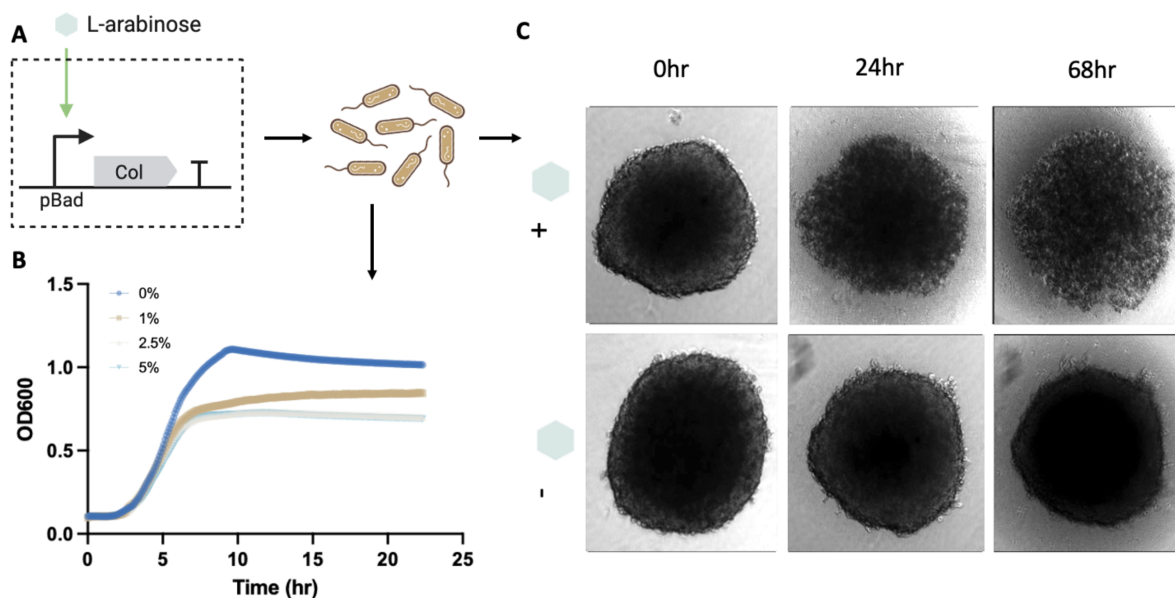


Figure 1.1. Arabinose-inducible collagenase expression is metabolically tolerated and produces progressive ECM disruption in HCT-116 spheroids. (A) Schematic of the pBad-Col construct. The collagenase gene (*col*) is under the control of the arabinose-inducible pBad promoter. L-arabinose (green hexagon) is added exogenously to induce expression. (B) Growth curves (OD₆₀₀ over 25 hours) of pBad-Col *S. typhimurium* at four arabinose concentrations: 0% (blue), 1% (tan), 2.5% (light grey), and 5% (light blue). Maximum OD decreases dose-dependently with increasing arabinose, consistent with metabolic burden, but growth is maintained at all concentrations. (C) Representative brightfield images of HCT-116 spheroids co-cultured with pBad-Col bacteria under uninduced (top row, 0% arabinose) or induced (bottom row, 5% arabinose) conditions at 0, 24, and 68 hours. Uninduced spheroids maintain compact architecture; induced spheroids show progressive ECM dissolution beginning at 24 hours and advancing to marked architectural disruption by 68 hours. Supplementary Videos 1 and 2 provide real-time visualization of the uninduced and induced conditions, respectively.

Figure 1.2A, left panel). For the collagenase strain, the *hlyE* therapeutic payload was replaced with the codon-optimized SOT gene (N-terminal flagellin fusion for outer membrane display), the lysis module was inactivated to generate a non-lytic, membrane-display variant (designated ST-pLux), and the reporter module was retained as a design reference but omitted from the final functional construct to reduce metabolic burden (Figure 1.2A, right panel). The resulting ST-pLux-Collagenase strain harbors the LuxI/LuxR quorum-sensing activator and membrane-anchored SOT under LuxR-AHL transcriptional control, so that collagenase expression is activated only when intratumoral bacterial density produces sufficient AHL to cross the quorum threshold. The behavioral consequence of this architecture is illustrated in the schematic below each circuit diagram: whereas the SLC produces growth → quorum sensing → synchronized lysis cycles (Figure 1.2A, lower left), the ST-pLux-Collagenase strain produces growth → quorum sensing → synchronized membrane-anchored enzyme expression without lysis (Figure 1.2A, lower right), enabling sustained collagenolytic activity from a living, proliferating bacterial population within the tumor core.

This design is motivated by two key considerations. First, removing the lysis module eliminates the population collapse that follows each SLC lysis cycle, allowing the collagenase-expressing population to maintain higher sustained biomass and therefore higher sustained enzyme output. Second, coupling collagenase expression to a quorum threshold rather than to exogenous arabinose induction makes enzyme production dependent on intratumoral bacterial density rather than on systemic administration of a small-molecule inducer, which would face substantial barriers to intratumoral bioavailability in a clinical context. The quorum-regulated system is therefore both more autonomous and more translationally rational than the pBad-Col proof-of-concept system.

1.2.4 Quorum-regulated collagenase activity is density-dependent and produces progressive ECM disruption in HCT-116 spheroids

To characterize the functional behavior of the ST-pLux-Collagenase strain in the HCT-116 BSCC model, we co-cultured bacteria with spheroids across three bacterial seeding densities, low, medium, and high CFU/well, and imaged spheroids by brightfield microscopy at a fixed timepoint of approximately 20 hours post-inoculation (Figure 1.2B). This seeding density series was designed to probe the density-dependence of collagenase activity, testing the prediction that higher bacterial density leads to faster quorum threshold attainment and therefore earlier and more extensive collagenase-mediated ECM disruption.

Three accompanying time-lapse videos capture the dynamic phenotype at each seeding density (Supplementary Video 3, low density; Supplementary Video 4, medium density; Supplementary Video 5, high density). The static images at the 20-hour timepoint and the corresponding videos reveal a striking density-dependent gradient of spheroid disruption. At low seeding density, the spheroid at 20 hours retains a largely intact outer matrix with visible peripheral bacterial accumulation but modest ECM disruption, consistent with bacteria still approaching the quorum threshold and collagenase expression only beginning (Figure 1.2B, left panel). At medium seeding density, the spheroid shows more pronounced disruption of the peripheral matrix with loss of outer ECM definition, reflecting earlier quorum attainment and sustained collagenase secretion at this timepoint (Figure 1.2B, center panel). At high seeding density, the spheroid at 20 hours exhibits the most dramatic phenotype: the peripheral matrix has been extensively degraded, the outer fibrous corona is largely dissolved, and the spheroid core appears exposed with markedly disrupted architecture (Figure 1.2B, right panel). Supplementary Video 5 is particularly striking at this density, capturing the real-time dissolution of the spheroid outer matrix in a pattern that visually resembles explosive radial disaggregation, a vivid demonstration of the ECM-degrading potency of the quorum-activated collagenase at sufficiently high bacterial burden.

The density-dependent collagenase phenotype is consistent with the expected behavior of a quorum-regulated circuit: at low density, the AHL concentration remains below the LuxR activation threshold and collagenase expression is minimal; as bacterial density increases toward and beyond the quorum threshold, LuxR-AHL complexes accumulate and drive collagenase transcription, with the rate and extent of ECM disruption scaling with the sustained enzyme output above threshold. This density-dependent behavior is an important functional validation of the quorum-sensing architecture, confirming that the LuxI/LuxR circuit is operational and that the collagenase payload is being expressed in a population density-dependent manner rather than constitutively. The observation that even the low-seeding-density condition shows detectable peripheral ECM disruption at 20 hours suggests that the quorum threshold is achievable under BSCC culture conditions across the density range tested, and that the gradient of phenotypic severity reflects quantitative differences in the rate and duration of quorum threshold attainment rather than a binary on/off circuit response.

1.2.5 Quorum-regulated collagenase expression enhances intratumoral bacterial penetration at physiologically relevant seeding densities

The high-seeding-density experiments in Figure 1.2B demonstrate that the ST-pLux-Collagenase strain is capable of producing dramatic ECM disruption, but the degree of spheroid dissolution observed at high bacterial burden likely exceeds what would be encountered during the initial colonization phase of therapeutic bacteria in a solid tumor *in vivo*. The most physiologically relevant context for assessing the functional benefit of collagenase-mediated ECM remodeling is therefore at low bacterial seeding density, where the colonizing population must actively penetrate an intact ECM scaffold to establish intratumoral residence. We therefore designed a follow-up experiment using a low seeding density of approximately 100 cells per spheroid to assess whether quorum-regulated collagenase expression enhances colonization efficiency under conditions that more closely model the early stages of tumor infiltration.

To enable visualization of bacteria at these low seeding densities where brightfield

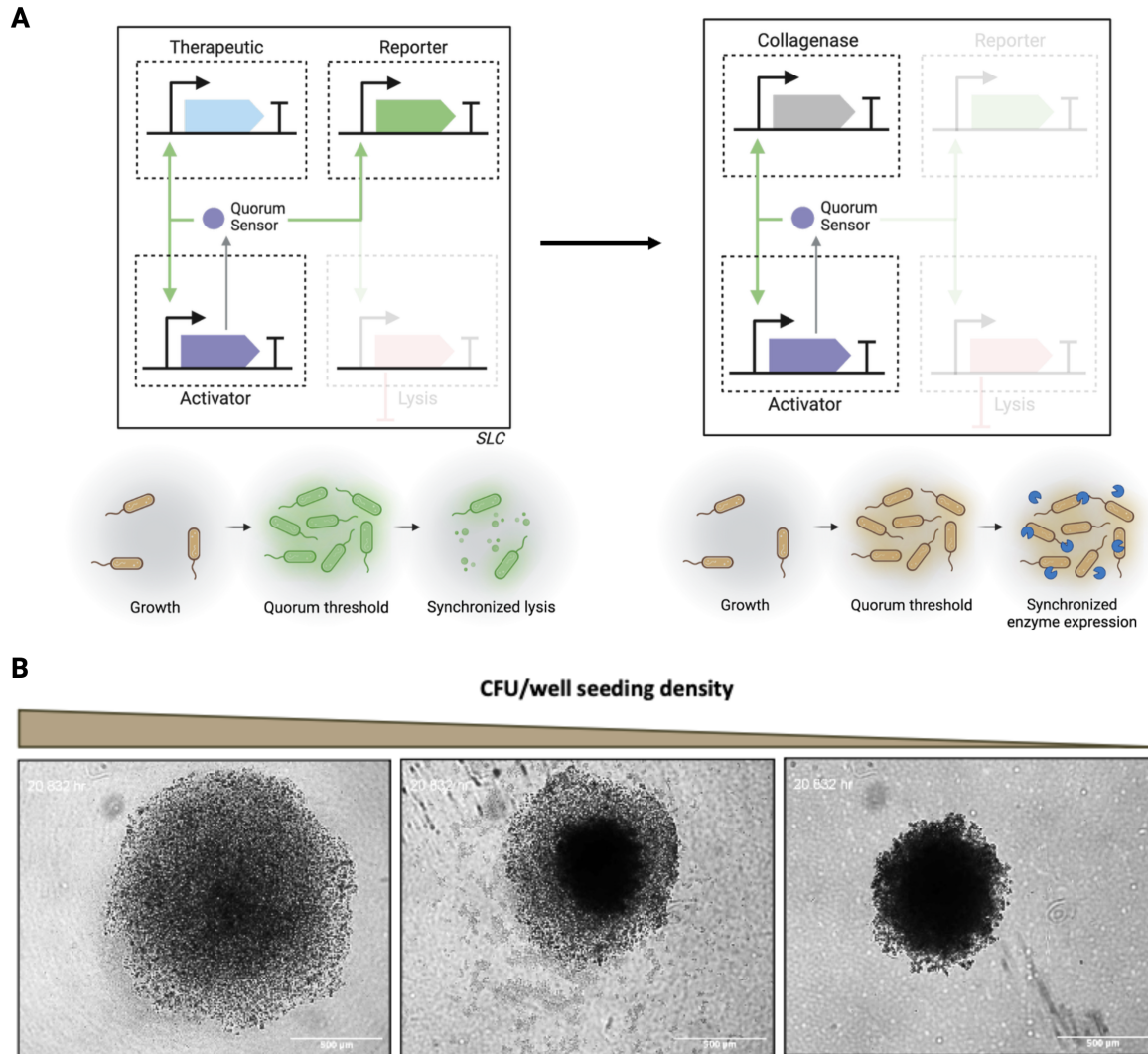


Figure 1.2. Quorum-regulated collagenase strain and density-dependent ECM disruption. (A) Circuit engineering schematic. Left: canonical SLC with *hlyE* therapeutic, *sfGFP* reporter, *luxI* activator, and ϕ X174E lysis modules (solid borders = active). Right: ST-pLux-Collagenase circuit with membrane-anchored SOT replacing *hlyE* and lysis module inactivated (dashed border). Schematics below illustrate resulting population behavior: SLC produces synchronized lysis cycles (left); ST-pLux-Collagenase produces synchronized membrane-anchored enzyme expression without collapse (right). (B) Representative brightfield images of HCT-116 spheroids co-cultured with ST-pLux-Collagenase at low (left), medium (center), and high (right) CFU/well seeding densities at approximately 20 hours post-inoculation. Scale bars, 500 μ m. ECM disruption is density-dependent. Supplementary Videos 3, 4, and 5 provide real-time visualization of low, medium, and high density conditions, respectively.

detection is insufficient, we introduced a GFP reporter into the collagenase strain, generating ST-pLux-Collagenase-GFP (Figure 1.3A). This strain retains the full quorum-regulated collagenase circuit (LuxI/LuxR activator, ST-pLux lysis-deficient backbone, membrane-anchored SOT) and additionally expresses constitutive GFP, enabling direct fluorescence-based quantification of intratumoral bacterial localization. The 1301-GFP parental strain (expressing GFP without collagenase) was used as the comparator control, and a no-bacteria vehicle control was included for baseline fluorescence normalization. All three conditions were assessed at the same low initial inoculation density, so that differences in GFP/tumor area signal reflect genuine differences in intratumoral penetration and colonization rather than input bacterial load.

Representative GFP fluorescence images of HCT-116 spheroids at 0 hours, 24 hours, and 5 days post-inoculation reveal a clear and progressive divergence between ST-pLux-Collagenase-GFP and 1301-GFP conditions (Figure 1.3B). At 0 hours, all three conditions appear dark with no detectable intraspheroidal GFP signal, consistent with bacteria not yet having penetrated the spheroid interior at the time of the first imaging point. At 24 hours, ST-pLux-Collagenase-GFP spheroids show distributed GFP puncta within the spheroid body, including signal extending toward the spheroid core, whereas 1301-GFP spheroids remain largely dark with only sparse peripheral signal. By 5 days, the difference is further accentuated: ST-pLux-Collagenase-GFP spheroids show bright, distributed intraspheroidal GFP signal indicating extensive bacterial colonization of the spheroid interior, while 1301-GFP spheroids show minimal fluorescence increase compared to the 5-day timepoint, and the no-bacteria control remains dark throughout.

Quantification of GFP fluorescence normalized to tumor spheroid area confirms the statistical significance of these differences at both timepoints (Figure 1.3C). At 24 hours, ST-pLux-Collagenase-GFP showed significantly higher GFP/tumor area than both the no-bacteria control ($**p < 0.01$) and 1301-GFP ($***p < 0.001$). At 5 days, ST-pLux-Collagenase-GFP again significantly outperformed both the no-bacteria control ($*p < 0.05$) and 1301-GFP ($*p < 0.05$). In contrast, 1301-GFP was not significantly different from the no-bacteria control at either timepoint, indicating that the parental strain without collagenase fails to achieve meaningful

intratumoral colonization above background at low seeding density over a 5-day window. The colonization advantage of ST-pLux-Collagenase-GFP over 1301-GFP is therefore attributable specifically to SOT-mediated ECM remodeling rather than to strain-intrinsic differences in motility or invasiveness, since both strains share the same 1301 chassis and differ only in the presence of the quorum-regulated SOT construct.

These results constitute the primary functional evidence that quorum-regulated collagenase expression enhances bacterial colonization efficiency in a three-dimensional tumor model. The fact that this advantage is detectable as early as 24 hours and is maintained through 5 days indicates that collagenase-mediated ECM disruption provides a durable and quantitative colonization benefit, not merely a transient facilitation of initial spheroid entry. The finding is particularly notable given the low seeding density employed, at approximately 100 cells per spheroid, the colonizing population must traverse a substantially intact ECM to reach the hypoxic core where conditions favor long-term bacterial residence, and the collagenase circuit appears to lower this physical barrier sufficiently to produce a measurable and statistically significant improvement in colonization outcome. These findings directly motivate the extension of the ST-pLux-Collagenase platform to physiologically relevant patient-derived organoid and in vivo contexts described in Aims 2 and 3.

1.3 Methods

1.3.1 Bacterial strains and plasmid construction

All experiments in this chapter used *Salmonella enterica* serovar Typhimurium strain ELH1301 (ST 1301) as the parental chassis [31]. This strain is an attenuated derivative suitable for biosafety level 2 handling and has been validated extensively for tumor spheroid co-culture applications. All bacterial handling was performed under BSL-2 conditions in a dedicated biosafety cabinet separate from tumor cell culture areas.

pBad-Col (arabinose-inducible collagenase) The gene encoding *Streptomyces omiyaen-*

sis trypsin (SOT), a bacterial serine protease with collagenolytic activity against collagens I and IV [15], was codon-optimized for expression in *S. typhimurium* 1301 prior to cloning. The codon-optimized SOT sequence was fused to an N-terminal flagellin signal sequence to facilitate surface display on the outer membrane of ST, as described in Ebelt et al. [15], and cloned downstream of the *araBAD* promoter (pBad) in a low-copy-number plasmid backbone (pBAD18-Cm, chloramphenicol resistance) using standard restriction-ligation cloning. The resulting pBad-Col plasmid was sequence-verified and transformed into electrocompetent ST 1301 cells. Transformants were selected on LB agar supplemented with 34 $\mu\text{g/mL}$ chloramphenicol, and surface display of SOT was confirmed by immunofluorescence staining against a C-terminal His-tag in arabinose-induced cultures.

ST-pLux-Collagenase (quorum-regulated collagenase) The quorum-regulated collagenase construct was derived from the synchronized lysis circuit (SLC) plasmid reported in Din et al. [12]. The therapeutic payload cassette (*hlyE*) was excised by restriction digestion and replaced with the codon-optimized SOT gene (N-terminal flagellin fusion for outer membrane display, as above) via Gibson Assembly, generating a construct in which membrane-anchored SOT expression is driven under LuxR-AHL transcriptional control. The lysis module (ϕX174E) was deleted, producing a strain designated ST-pLux-Collagenase that accumulates at quorum and sustains membrane-displayed collagenolytic activity without undergoing synchronized population collapse. The GFP reporter cassette was retained in the circuit architecture as a design reference but was omitted from the functional construct to minimize metabolic burden. The resulting ST-pLux-Collagenase plasmid was sequence-verified across all junctions and confirmed for SOT expression by induction of high-density liquid cultures with exogenous AHL (10 nM N-(β -ketocaproyl)-L-homoserine lactone; Sigma K3007) followed by immunofluorescence staining against the C-terminal His-tag.

ST-pLux-Collagenase-GFP (quorum-regulated collagenase with GFP reporter) To enable fluorescence-based quantification of bacterial colonization at low seeding densities where brightfield detection is insufficient, a quorum-regulated sfGFP was introduced downstream

of collagenase and pLux promoter on the same plasmid. GFP expression was confirmed by spectrophotometry (not shown) and fluorescence microscopy.

1301-GFP (parental control) ST 1301 was transformed with the pLux-GFP plasmid alone, providing a matched comparator for the colonization experiments in Figure 1.3.

1.3.2 Bacterial culture conditions

Bacteria were cultured from glycerol stocks by inoculation into LB broth (Lennox; Thermo Fisher) supplemented with appropriate selection antibiotics (chloramphenicol 34 $\mu\text{g/mL}$ for pBad-Col; kanamycin 50 $\mu\text{g/mL}$ for GFP-expressing strains; both where applicable). Overnight cultures were grown at 37°C with 200–250 rpm orbital shaking for 12–16 hours in dual-position snap-cap culture tubes to allow aeration. On the day of co-culture, overnight cultures were measured by OD₆₀₀ on a NanoDrop OneC spectrophotometer, then adjusted to OD₆₀₀ = 0.5 by dilution with sterile 1× DPBS (equivalent to approximately $1\text{--}2 \times 10^8$ CFU/mL for ST 1301 [31]). For the BSCC inoculation, 2 μL of this suspension was added per spheroid well, yielding approximately $10^5\text{--}10^6$ CFU per spheroid at standard density, or approximately 100 cells per spheroid at the low-density condition used in Figure 1.3.

For arabinose induction experiments, L-arabinose (Sigma) was dissolved in sterile water and added directly to the co-culture medium at the time of bacterial inoculation at the indicated concentrations (0%, 1%, 2.5%, 5% w/v). Bacterial growth curves under arabinose induction were measured in flat-bottom 96-well plates in a BioTek Synergy H1 multimode plate reader at OD₆₀₀ with 15-minute measurement intervals over 24 hours at 37°C with continuous orbital shaking.

1.3.3 HCT-116 tumor spheroid preparation

HCT-116 human colorectal carcinoma cells (ATCC CCL-247) were maintained in McCoy's 5A medium (Gibco) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco) and 1% (v/v) penicillin-streptomycin at 37°C in 5% CO₂. Cells were passaged at

70–80% confluency using 0.05% trypsin-EDTA.

Tumor spheroids were generated using the ultra-low attachment (ULA) round-bottom 96-well plate method [31]. Cells were trypsinized, counted by hemocytometer with trypan blue exclusion, and resuspended in spheroid growth medium (McCoy's 5A, 10% FBS, without antibiotics) at a density of 2500 cells per 100 μ L. Using a multichannel pipette, 100 μ L of cell suspension was transferred into each well of a Corning Costar Ultra-Low Attachment 96-well round-bottom plate (Sigma CLS7007). The plate was centrifuged at 100 $\times$ g for 5 minutes to consolidate cells at the well bottom, then transferred to a 37°C CO₂ incubator. Spheroids were allowed to form and develop a hypoxic necrotic core over 4 days, with daily brightfield monitoring to confirm spheroid integrity and necrotic center formation. Spheroids were used for co-culture on day 4 when a darker, denser core was visible by brightfield microscopy, indicating establishment of the oxygen and nutrient gradients that support intratumoral bacterial colonization.

1.3.4 Bacteria-spheroid co-culture (BSCC) protocol

The BSCC protocol was performed as described in Harimoto, Deb, and Danino [?], with modifications for the ST 1301 strain and collagenase-expressing constructs. On day 4 of spheroid culture, bacterial suspensions were prepared as described above and 2 μ L of OD₆₀₀-adjusted suspension was added to each spheroid well. Plates were returned to a dedicated 37°C CO₂ incubator separate from the tumor cell incubator and incubated for 2 hours to allow initial bacterial attachment and infiltration into the spheroid periphery. Following the 2-hour incubation, extratumoral bacteria were removed by a series of medium exchange washing steps: 60 μ L of medium was aspirated from each well using a p200 pipette angled at 45° to avoid disturbing the spheroid, 160 μ L of sterile 1 $\times$ DPBS was gently added along the well side, and the aspiration step was repeated, for a total of three complete wash cycles. After washing, 200 μ L of co-culture medium (McCoy's 5A, 10% FBS, 2.5 μ g/mL gentamicin) was added to each well. Gentamicin at this concentration kills extratumoral bacteria in the surrounding medium while failing to diffuse

at bactericidal concentrations into the spheroid core, selectively confining bacterial growth to the intratumoral environment [?]. Co-cultures were maintained at 37°C with medium changes every 3–4 days for the duration of each experiment.

For the seeding density series in Figure 1.2B, three bacterial input densities were tested by adjusting the OD₆₀₀ of the inoculation suspension prior to the 2 μ L addition: low (~ 100 CFU/well), medium ($\sim 10^4$ CFU/well), and high ($\sim 10^6$ CFU/well), spanning approximately four orders of magnitude. All other protocol steps were identical across density conditions.

1.3.5 Brightfield and fluorescence imaging

All imaging was performed on a Nikon Ti-E microscope equipped with brightfield, phase contrast, and GFP fluorescence (excitation 469/35 nm, emission 525/39 nm) cubes. Spheroids were imaged in situ within the 96-well ULA plate at 4 $\times$ magnification. For time-lapse experiments (Supplementary Videos 1–5), plates were transferred to an Okolab stage-top incubator maintaining 37°C, 5% CO₂, and 95% humidity, and images were acquired every 60 minutes over the indicated time periods using the Nikon Elements software as described in the BSCC protocol [31]. Images were exported as uncompressed 16-bit TIFFs for downstream analysis.

GFP fluorescence within spheroids was quantified using a custom ImageJ macro. Briefly, brightfield images were thresholded using the Yen method to generate a binary mask defining the spheroid boundary; the corresponding GFP channel image was then measured within this mask to obtain integrated fluorescence intensity, which was subsequently normalized to the masked spheroid area (GFP a.u./tumor area). All quantifications were performed with identical hardware settings and image acquisition parameters across all conditions and timepoints within each experiment.

1.3.6 Time-lapse video acquisition and processing

Time-lapse videos were generated from brightfield image stacks acquired every 60 minutes on the Nikon TiA microscope equipped with an Okolab stage-top incubator [31]. Image stacks were assembled into video files using FIJI (ImageJ) with the Bio-Formats importer. Each frame represents one 5-minute imaging interval; videos are presented at 15 frames per second to allow visualization of the full temporal dynamics of spheroid and bacterial behavior. Five videos are provided as supplementary materials:

- **Supplementary Video 1:** pBad-Col, uninduced (0% arabinose) — HCT-116 spheroid morphology over 68 hours, demonstrating stable spheroid architecture in the absence of collagenase induction.
- **Supplementary Video 2:** pBad-Col, induced (5% arabinose) — HCT-116 spheroid over 68 hours, demonstrating progressive ECM disruption and matrix dissolution following collagenase induction.
- **Supplementary Video 3:** ST-Collagenase, low seeding density — HCT-116 spheroid co-culture at low CFU/well input, showing modest peripheral ECM disruption consistent with approach to quorum threshold.
- **Supplementary Video 4:** ST-Collagenase, medium seeding density — HCT-116 spheroid co-culture at intermediate CFU/well input, showing intermediate ECM disruption phenotype with accelerated matrix dissolution relative to low density.
- **Supplementary Video 5:** ST-Collagenase, high seeding density — HCT-116 spheroid co-culture at high CFU/well input, showing rapid and extensive spheroid matrix dissolution with dramatic radial disaggregation of the outer ECM corona.

1.3.7 Statistical analysis

Statistical comparisons of GFP fluorescence/tumor area between conditions (Figure 1.3C) were performed using one-way ANOVA with Tukey's multiple comparisons post-hoc test in GraphPad Prism v9. Normality was assessed by Shapiro-Wilk test; one-way ANOVA was applied where normality assumptions were met. All experiments include at least three independent spheroid replicates per condition. Data are presented as mean $\pm$ SEM. Significance thresholds are denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ throughout all figures and text.

1.4 Acknowledgements

Chapter 1, in part, is currently being prepared for submission for publication of the material. The dissertation author was the primary investigator and author of this material.

1.5 Supplementary Materials

Supplementary Videos 1–5 are provided as separate video files and accompany this chapter. Video file formats are .mp4 (H.264 encoding). All videos were acquired at 5-minute intervals and are presented at 15 frames per second.

- **Supplementary Video 1:** ST-pBad-Col uninduced (0% arabinose), 68-hour time-lapse.
- **Supplementary Video 2:** ST-pBad-Col induced (5% arabinose), 68-hour time-lapse.
- **Supplementary Video 3:** ST-Collagenase, low seeding density.
- **Supplementary Video 4:** ST-Collagenase, medium seeding density.
- **Supplementary Video 5:** ST-Collagenase, high seeding density.

Chapter 2

A Patient-derived Organoid Platform for Bacterial Chassis Evaluation

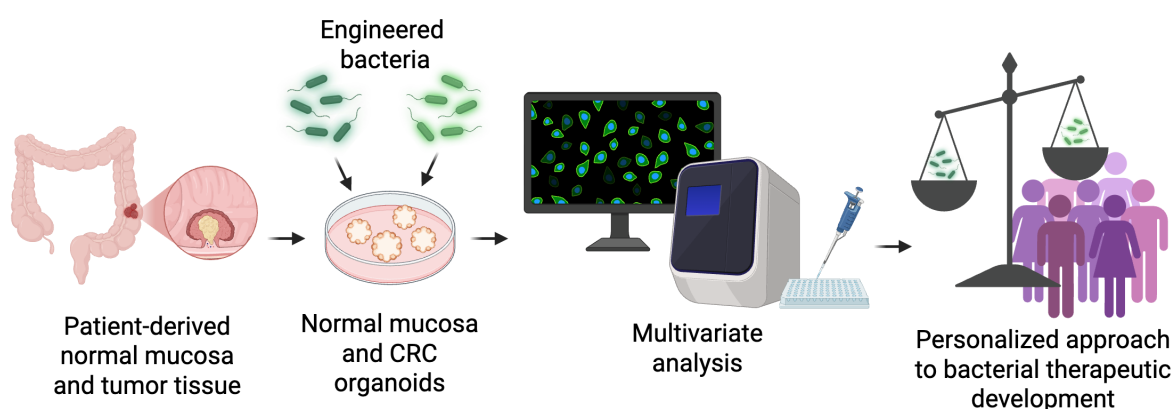


Figure 2.1. Overview of the patient-derived organoid co-culture platform. Patient-derived normal mucosa and tumor tissue from CRC surgical resections are used to establish matched normal colon and CRC organoid lines. Both organoid types are co-cultured with engineered bacteria and subjected to multivariate analysis including fluorescence imaging, morphometric quantification, CFU enumeration, and transcriptional profiling, enabling a personalized approach to bacterial therapeutic development.

2.1 Summary

Standard preclinical models for evaluating bacterial cancer therapeutics rely heavily on immortalized cell lines that fail to recapitulate the genetic heterogeneity, architectural complexity, and patient-specific biology of clinical colorectal carcinoma (CRC). To address this gap,

this chapter describes the establishment and systematic characterization of a patient-derived organoid (PDO) co-culture platform in which matched tumor and normal colon organoids from two CRC patients, representing microsatellite-stable (MSS) and microsatellite-unstable (MSI) molecular subtypes, were co-cultured with *Salmonella typhimurium* 1301 (ST) and *Escherichia coli* Nissle 1917 (EcN), two leading engineered bacterial therapeutic chassis. Prior to organoid co-culture, both strains were characterized under a panel of CRC-relevant tumor microenvironment (TME) chemical stressors to establish baseline fitness comparisons under physiologically relevant conditions. In matched PDO co-cultures, ST achieved superior colonization efficiency, tumor specificity, and sustained intratumoral persistence compared to EcN across all measured readouts, including colony-forming unit (CFU) enumeration, GFP mean fluorescence intensity (MFI), and time-lapse colonization kinetics. Bacterial colonization burden was coupled to a distinct and reproducible set of organoid morphological changes, quantified through a 31-feature computational pipeline; integrated density and raw integrated density emerged as top fluorescence-independent classifiers of high-colonization state, with area under the receiver operating characteristic curve (AUC) values of 0.971. ST exposure also drove strain-specific transcriptional remodeling across inflammatory, metabolic, and epithelial-to-mesenchymal transition (EMT) programs, and reduced secondary organoid-forming capacity relative to PBS and EcN controls. Cross-patient conservation analyses revealed that morphological and transcriptional response signatures were broadly maintained between MSS and MSI lines, with attenuated effect sizes in the MSI patient consistent with known therapeutic resistance in that subtype. Together, these results establish a validated, multi-modal PDO platform for evaluating bacterial therapeutic candidates with patient-stratified resolution, and provide the methodological foundation for fluorescence-independent colonization assessment applied to the untagged ST-pLux-Collagenase strain in Aim 3.

2.2 Results

2.2.1 *S. typhimurium* and *E. coli* Nissle 1917 exhibit differential fitness under CRC-relevant tumor microenvironment chemical stressors

The tumor microenvironment of solid CRC lesions is defined by a constellation of biochemical stressors that differ markedly from the conditions encountered in standard laboratory culture [30, 23]. Hypoxia arising from inadequate vascularization, acidosis resulting from elevated anaerobic glycolysis, reactive oxygen species (ROS) generated by inflammatory infiltrates, and elevated osmolarity from tumor interstitial fluid accumulation are collectively hostile to many therapeutic agents, yet may be selectively tolerated or even exploited by facultative anaerobic bacteria such as ST [61, 12]. Before deploying ST and EcN in the organoid co-culture system, we therefore characterized both strains under a systematic panel of these TME-relevant chemical stressors to establish their differential physiological fitness profiles and identify conditions under which their colonization behavior might diverge.

GFP-expressing derivatives of both strains (ST-GFP and EcN-GFP) were subjected to gradient screening of four stressor categories: oxygen tension (0%, 10.5%, and 21.5% O₂), pH (5.9, 6.88, and 7.4), hydrogen peroxide as a ROS proxy (0, 0.2, and 0.6 mM H₂O₂), and sodium chloride as an osmolarity surrogate (0.3, 0.36, and 0.43 M NaCl). Growth rate (OD-normalized, 1/s) and maximum GFP fluorescence per OD unit (Max GFP/OD, a proxy for metabolic and translational activity) were quantified across all conditions in a microplate reader format (Figure 2.2).

Under oxygen deprivation, both strains exhibited significantly elevated growth rates relative to normoxic controls, consistent with their facultative anaerobic metabolic capacity, but ST-GFP achieved markedly higher growth rates across all oxygen conditions than EcN-GFP (**** $p < 0.0001$; Figure 2.2C). The coefficient of variance (CoV) for growth rate under oxygen stressor was substantially higher for EcN (CoV ≈ 0.12) than ST (CoV ≈ 0.11), indicating greater phenotypic instability in EcN under hypoxic challenge. For fluorescence output, ST-GFP

displayed a strikingly high CoV under oxygen stressor (≈ 0.34) compared to all other conditions, reflecting disproportionate GFP expression variance under low-oxygen conditions that may reflect metabolic state heterogeneity within the ST population (Figure 2.2D).

Under acidic pH conditions, growth rates were not significantly different between strains at physiological or mildly acidic pH (pH 6.88 vs. 7.4: ns; Figure 2.2C), though both strains showed modestly reduced growth at pH 5.9. GFP output was similarly unaffected by pH variation for both strains (ns). These findings suggest that the moderate acidosis typical of the CRC TME (pH ~ 6.5 – 7.0) does not constitute a major fitness barrier for either strain.

ROS exposure revealed a divergent pattern: both strains exhibited significantly reduced growth rates at 0.6 mM H₂O₂ compared to lower concentrations (**** $p < 0.0001$), but the magnitude of suppression was more pronounced for EcN-GFP. At the GFP output level, ST-GFP showed a significant increase in Max GFP/OD at 0.6 mM relative to 0.2 mM ROS (** $p < 0.01$), suggesting an oxidative stress-response induction, while EcN-GFP was unaffected (ns). EcN-GFP exhibited higher ROS CoV for growth rate (≈ 0.24) than ST-GFP (≈ 0.04), further underscoring the greater fitness variance of EcN under oxidative challenge (Figure 2.2C–D).

Under hyperosmolar NaCl conditions, both strains displayed significantly reduced growth rates at 0.43 M compared to 0.3 M (**** $p < 0.0001$; Figure 2.2C), consistent with osmotic stress-induced growth inhibition. GFP output for both strains was significantly affected by NaCl concentration (**** $p < 0.0001$), with ST-GFP maintaining substantially higher Max GFP/OD across conditions. The GFP/OD CoV for NaCl was among the lowest across all stressors for both strains (ST ≈ 0.05 , EcN ≈ 0.05 ; Figure 2.2D), indicating that osmolarity effects on translational output were relatively uniform within each population.

Taken together, these data indicate that ST exhibits consistently superior growth rate and fluorescent protein expression output across the full panel of CRC TME chemical stressors, with particularly notable advantages under hypoxic and oxidative conditions. EcN displays greater fitness variance under the most physiologically challenging conditions, suggesting lower phenotypic robustness in the TME chemical niche that bacterial therapeutics must navigate

post-colonization. These differential fitness profiles provide important context for interpreting the superior colonization outcomes of ST in the organoid co-culture experiments described below.

2.2.2 ST achieves superior colonization efficiency and tumor specificity in patient-derived CRC organoids

To evaluate bacterial colonization in a patient-representative model of CRC, we established a PDO co-culture platform using tissue resected from two CRC patients: a microsatellite-stable (MSS) primary line and a microsatellite-unstable (MSI) primary line. Matched normal colon (NC) organoids from uninvolved mucosa adjacent to the tumor were established in parallel for each patient to enable direct tumor-versus-normal specificity comparisons. The experimental workflow involved culturing PDOs in antibiotic-free advanced DMEM/F12 medium for 24 hours prior to inoculation, followed by addition of ST-GFP, EcN-GFP, or PBS vehicle control at a standardized input density, with subsequent analysis by fluorescence microscopy, CFU quantification, and multi-parametric computational readouts at 24, 48, and 72 hours post-inoculation (Figure 2.3A).

Phase-contrast and GFP fluorescence microscopy at 48 hours post-inoculation revealed qualitatively distinct colonization phenotypes between strains and organoid types. In MSS CRC organoids, ST-GFP produced intense, diffuse GFP signal concentrated within and immediately surrounding organoid bodies, whereas EcN-GFP generated only sparse punctate fluorescence and PBS-treated organoids showed no detectable GFP (Figure 2.3B). In matched NC organoids, both strains produced dramatically reduced fluorescence compared to CRC counterparts, with ST-GFP generating only low-level peripheral signal and EcN-GFP remaining essentially dark, indicating preferential colonization of tumor tissue (Figure 2.3B).

Quantitative CFU enumeration from homogenized wells at 48 hours confirmed the visual observations. ST-GFP colonized MSS CRC organoids at significantly higher bacterial load than EcN-GFP ($***p < 0.001$), and both strains showed significantly elevated CFU in CRC

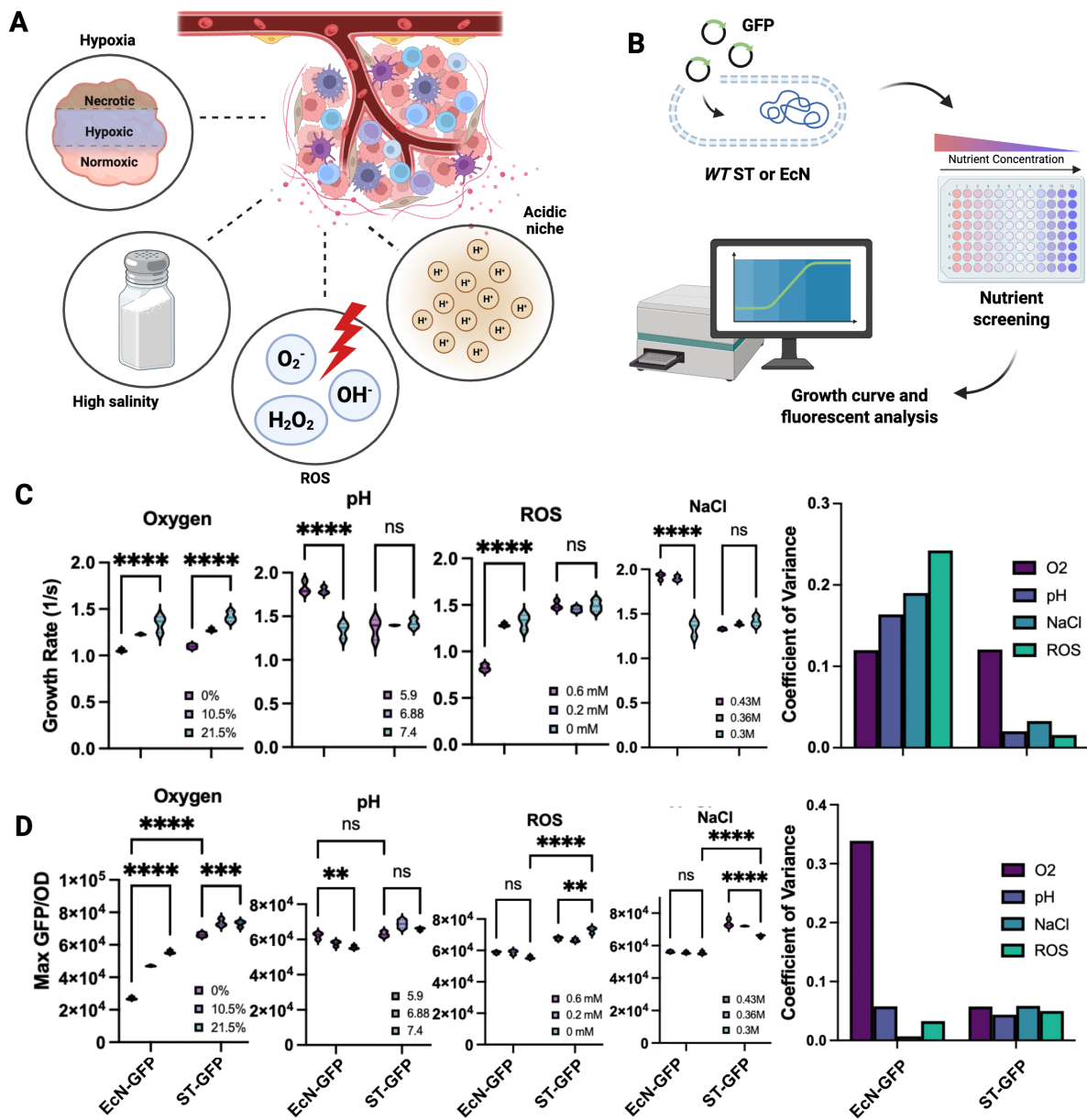


Figure 2.2. ST and EcN exhibit differential fitness under CRC-relevant TME chemical stressors. (A) Schematic of the four TME stressor categories: hypoxia, acidic niche, reactive oxygen species (ROS), and high salinity. (B) Batch culture gradient screening workflow. GFP-expressing ST or EcN are subjected to stressor concentration gradients in 96-well plates; growth and fluorescence are monitored by plate reader. (C) Growth rate (OD₆₀₀-normalized, 1/s) for EcN-GFP and ST-GFP across oxygen tension, pH, ROS, and NaCl gradient conditions. Significance brackets: ns, *** $p < 0.001$, **** $p < 0.0001$. Bar chart (right): coefficient of variance for growth rate across stressor categories. (D) Maximum GFP fluorescence per OD unit (Max GFP/OD) across the same stressor panel. Bar chart (right): coefficient of variance for Max GFP/OD. ST-GFP shows consistently superior growth rates and GFP output, with particularly high GFP/OD variance under oxygen stress.

compared to matched NC organoids (ST: $**p < 0.01$; EcN: $**p < 0.01$; Figure 2.3C). The magnitude of ST colonization was approximately 5-fold higher than EcN in CRC organoids, and the CRC-to-NC selectivity ratio was substantially greater for ST than EcN, indicating not only quantitative superiority but also greater tumor specificity. A strain-by-tissue interaction was observed in which ST-GFP CRC colonization significantly exceeded PBS and EcN-GFP controls ($***p < 0.001$), while EcN-GFP CRC colonization, though statistically elevated above NC, remained quantitatively modest (Figure 2.3C).

Time-lapse GFP MFI normalized to tumor organoid area over 72 hours post-inoculation revealed dynamic colonization kinetics consistent with the static endpoint CFU data. ST-GFP showed a rapid increase in MFI/tumor area between 0 and 48 hours, peaking at approximately 40 MFI units at 48 hours before declining modestly by 72 hours, while EcN-GFP and PBS remained near zero throughout (Figure 2.3D). The corresponding colonization rate (MFI/hr) was approximately 0.25 for ST-GFP versus near-zero for both comparators, confirming that ST establishes rapidly expanding intratumoral colonies while EcN fails to achieve sustained colonization under the co-culture conditions employed (Figure 2.3D, inset).

In the MSI patient line, the directional trends were conserved: ST-GFP achieved significantly higher CFU in CRC relative to NC ($****p < 0.0001$) and significantly outperformed EcN-GFP ($****p < 0.0001$), and time-lapse MFI kinetics showed the same ST-specific colonization dynamic (Figure 2.8A–B). However, absolute colonization magnitudes were approximately one order of magnitude lower in the MSI line across all conditions, consistent with the known therapeutic resistance profile of this patient's tumor and the reduced permissivity of MSI CRC to bacterial infiltration observed in broader clinical contexts [?]. This attenuation is interpreted as a biologically meaningful patient-specific phenotype rather than a platform failure, and it underscores the capacity of the PDO system to resolve inter-patient differences in bacterial colonization susceptibility.

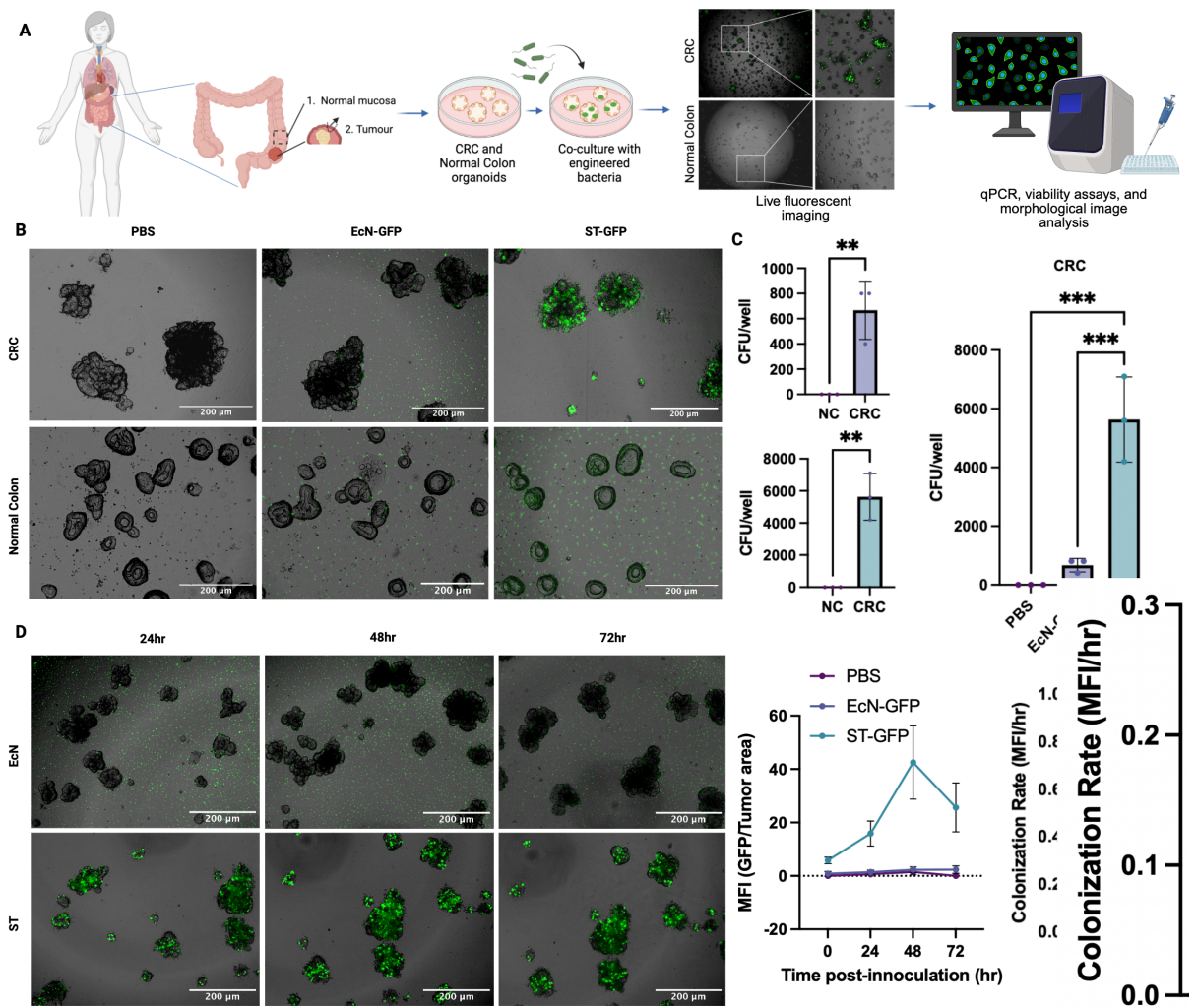


Figure 2.3. ST achieves superior colonization efficiency and tumor specificity in patient-derived CRC organoids. (A) Schematic of the PDO co-culture workflow. (B) Representative composite fluorescence and brightfield images of MSS CRC (top) and matched NC (bottom) organoids at 48 hours with PBS, EcN-GFP, or ST-GFP. Scale bars, 200 μ m. (C) Left: CFU/well at 48 hours for NC and CRC organoids inoculated with EcN-GFP or ST-GFP. Both strains show significantly higher CFU in CRC vs NC (** $p < 0.01$). Right: CFU/well across all conditions in CRC organoids; ST-GFP significantly exceeds EcN-GFP and PBS (***) $p < 0.001$. (D) Left: representative composite images of CRC organoids with EcN (top) or ST (bottom) at 24, 48, 72 hours. Scale bars, 200 μ m. Center: GFP MFI/tumor area over 72 hours for PBS (purple), EcN-GFP (dark blue), ST-GFP (teal); ST-GFP peaks at 48 hours while comparators remain near zero. Right: colonization rate (MFI/hr) by condition.

2.2.3 Bacterial colonization burden is coupled to distinct morphological phenotypes in CRC organoids

Fluorescence-based quantification of bacterial colonization, while informative, is fundamentally dependent on the stable expression and retention of a fluorescent reporter gene in the colonizing strain. This presents a practical constraint for evaluating the collagenase-expressing ST-pLux-Collagenase strain used in Aim 3, which does not carry a GFP transgene, and motivated the development of a fluorescence-independent colonization readout based on organoid morphological features. We hypothesized that high-level ST colonization would impose measurable structural and optical changes on organoid bodies, reflecting altered metabolic state, cell death, cytoskeletal reorganization, and ECM interaction, that could be detected and quantified through brightfield image analysis.

To test this hypothesis, we extracted a 31-feature morphological profile from individual organoids in the MSS PDO co-culture dataset using a custom ImageJ/FIJI macro pipeline applied to brightfield images. Features spanned three functional domains: viability-associated metrics (area, mean grey value, modal grey value, min/max grey value, integrated density, raw integrated density), shape regularity metrics (perimeter, centroid coordinates, center of mass coordinates, center of mass deviation, major/minor axis, angle, circularity, min/max Feret diameter, percent area, Feret X/Y/angle, aspect ratio, roundness), and uniformity metrics (skew, solidity, standard deviation, coefficient of variance). Representative brightfield, GFP, and composite images at 100 μm scale confirm the visual correlates of this morphological stratification (Figure 2.4A).

Pairwise Spearman correlation analysis across all 31 features revealed three natural feature superclusters corresponding to the three functional domains, with strong intra-cluster correlations and attenuated inter-cluster relationships (Figure 2.4B). A feature-by-condition correlation heatmap against GFP MFI demonstrated that viability-domain features (particularly integrated density, raw integrated density, and grey value statistics) showed the strongest negative correlations with increasing MFI, while shape regularity features showed predominantly negative

correlations of intermediate magnitude, and uniformity features exhibited mixed directionality (Figure 2.4C).

Dimensionality reduction via UMAP projections applied to all 31 features produced clear condition-level separation, with ST-GFP organoids occupying a distinct region of embedding space from EcN-GFP and PBS organoids, which clustered together (Figure 2.4D, left). Overlay of GFP MFI as a continuous variable onto the same UMAP revealed a smooth MFI gradient that closely tracked the condition-level separation, confirming that the morphological feature space encodes colonization-associated signal independent of any fluorescence input (Figure 2.4D, right).

Linear regression of individual morphological features against per-organoid GFP MFI identified integrated density ($R^2 = 0.51$), standard deviation ($R^2 = 0.44$), skew ($R^2 = 0.50$), and solidity ($R^2 = 0.30$) as features with the strongest individual predictive relationships with colonization burden, while area showed a moderate relationship ($R^2 = 0.30$; Figure 2.4E). All significant regressions were negative (higher MFI associated with lower feature values), consistent with colonization-associated organoid shrinkage, darkening, and structural disruption.

Violin plot comparisons confirmed that these features were significantly different across all three conditions at the population level. ST-GFP organoids exhibited significantly reduced area, integrated density, standard deviation, and solidity compared to both EcN-GFP and PBS (**** $p < 0.0001$), while skew was significantly elevated in ST-GFP relative to comparators (**** $p < 0.0001$; Figure 2.4F). EcN-GFP organoids showed intermediate values for most features, with statistically significant differences from PBS in some metrics but effect sizes substantially smaller than those for ST-GFP.

Receiver operating characteristic (ROC-AUC) analysis of all morphological features as binary classifiers of high-colonization state (ST-GFP vs. PBS + EcN-GFP) demonstrated that raw integrated density and integrated density were the top-performing features with AUC = 0.971 each, followed by standard deviation (AUC = 0.935), skew (AUC = 0.931), mean grey value (AUC = 0.922), and modal grey value (AUC = 0.904; Figure 2.10). Size-related shape regularity

features (minor axis, area, major axis, min/max Feret, perimeter) achieved AUCs in the range of 0.82–0.89, while shape descriptors such as circularity (AUC = 0.787) and aspect ratio (AUC = 0.538) showed lower discriminatory power. The discriminatory power of integrated density as a colonization classifier (AUC = 0.971) provides the methodological basis for fluorescence-independent colonization assessment in Aim 3, where the untagged ST-pLux-Collagenase strain is evaluated using morphological profiling as a surrogate readout.

In the MSI line, the overall morphological response structure was directionally conserved: UMAP again produced condition-level separation with ST-GFP occupying a distinct embedding region, viability-domain features correlated most strongly with MFI, and ST-GFP organoids exhibited significantly reduced area and integrated density relative to comparators (Figure 2.9). However, individual regression R^2 values were substantially reduced across all features (area: $R^2 = 0.06$; integrated density: $R^2 = 0.07$; skew: $R^2 = 0.02$; solidity: $R^2 = 0.21$), and the linear relationships between MFI and morphological features were weaker and noisier, consistent with the lower absolute colonization magnitudes in this patient line.

2.2.4 Cross-patient conservation of morphological responses reveals conserved effect directionality with subtype-specific attenuation

To formally quantify the degree to which morphological responses to ST colonization are conserved versus patient-specific, we performed a cross-patient effect size conservation analysis comparing Cohen's d values for each morphological feature between the MSS and MSI lines under the ST-GFP versus PBS contrast (Figure 2.6A–B).

Across the 20 features with sufficient effect size to be interpretable, MSS and MSI Cohen's d values were significantly correlated ($r = 0.606$, $p = 0.006$), confirming that the directional structure of the morphological response is broadly conserved between patient lines (Figure 2.6A). Viability-domain features (integrated density, raw integrated density, grey value statistics) showed the largest and most consistent negative effect sizes in both lines, reflecting colonization-associated darkening and shrinkage that is robust across genetic subtypes. Shape

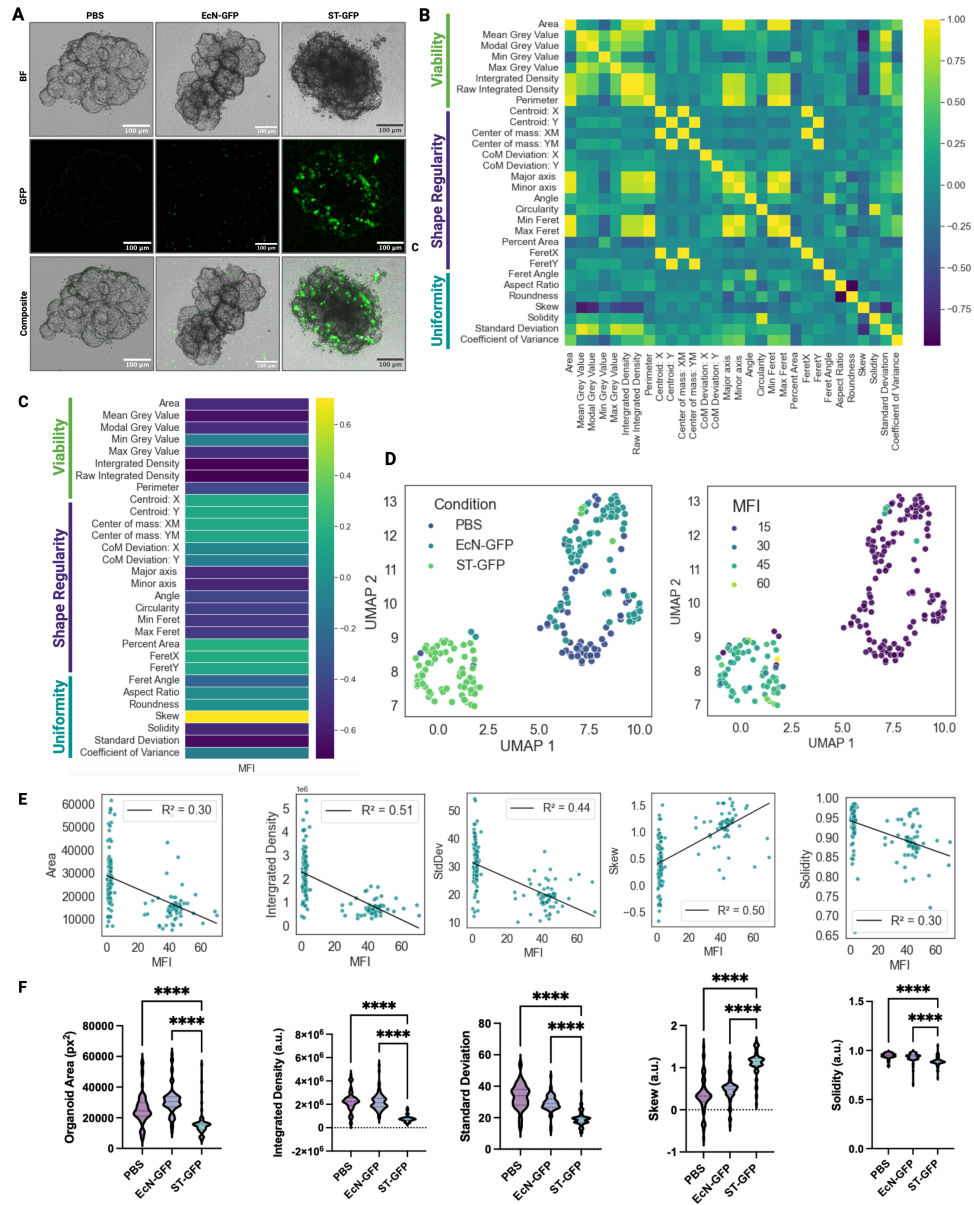


Figure 2.4. Bacterial colonization burden is coupled to distinct morphological phenotypes in CRC organoids. (A) Representative BF (top), GFP (middle), and composite (bottom) images of MSS CRC organoids at 48 hours for PBS, EcN-GFP, and ST-GFP. Scale bars, 100 μm . (B) Pairwise Spearman correlation matrix of all 31 morphometric features, grouped by functional domain. (C) Heatmap of Spearman correlations between each morphometric feature and GFP MFI. Viability-domain features show the strongest negative correlations. (D) UMAP projection colored by condition (left) and continuous GFP MFI (right). ST-GFP forms a distinct cluster; MFI gradient tracks condition separation. (E) Linear regression of five representative features against GFP MFI; R^2 values shown. (F) Violin plots of the same five features by condition. All pairwise comparisons significant (**** $p < 0.0001$).

regularity features (solidity, circularity) showed large, conserved negative effects (i.e. ST-GFP organoids are consistently less solid and less circular than PBS controls in both patients) while uniformity features showed more variable conservation status.

Attenuation ratio analysis ($\text{—MSI } d\text{—} / \text{—MSS } d\text{—}$) quantified the degree to which effect sizes were preserved versus dampened in the MSI line (Figure 2.6B). The majority of features fell in the “Conserved” category (attenuation ratio 0.5–1.0 $\times$), indicating that the MSI line recapitulates the directionality and approximately half or more of the magnitude of the MSS response. Several viability features (integrated density, raw integrated density, max/mean/modal grey values, standard deviation) showed attenuation ratios in the range of 0.1–0.4 $\times$, classified as “Attenuated,” consistent with the lower absolute colonization burden in the MSI line reducing the magnitude of viability-domain changes. Critically, coefficient of variance showed a markedly divergent response (attenuation ratio $> 1\times$, labeled as “Divergent”), indicating that intratumoral heterogeneity in the MSI line responds in the opposite direction to that observed in the MSS line. This divergence may reflect the distinct mutational architecture of MSI CRC, in which intratumoral heterogeneity is already high at baseline due to mismatch repair deficiency, and ST colonization may paradoxically reduce variance by imposing selective cytotoxic pressure. Two features (roundness, aspect ratio) were classified as “Not significant” in the MSI line, indicating that their MSS-line responses did not generalize to this patient.

These analyses support the interpretation that the PDO platform captures a biologically reproducible core morphological response to ST colonization that generalizes across MSS and MSI molecular subtypes, while also resolving patient-specific differences in response magnitude and the behavior of specific feature subsets. This dual capacity, for both inter-patient conservation and patient-specific stratification, positions the platform as a tool for both mechanistic discovery and translational precision medicine applications.

2.2.5 ST colonization drives strain-specific transcriptional remodeling in CRC organoids

To characterize the transcriptional consequences of ST and EcN colonization in patient-derived CRC organoids, we performed quantitative RT-PCR analysis on a seven-gene panel selected to capture key biological processes relevant to bacterial-tumor interactions: tumor necrosis factor alpha (*TNF*) as an indicator of inflammatory activation; lipocalin-2 (*LCN2*) as a marker of innate immune and epithelial stress response; pyruvate dehydrogenase kinase 4 (*PDK4*) as a metabolic reprogramming indicator; snail family transcriptional repressor 1 (*SNAIL*) and vimentin (*VIM*) as EMT markers; leucine-rich repeat-containing G protein-coupled receptor 5 (*LGR5*) as an intestinal stem cell marker; and mucin 2 (*MUC2*) as an indicator of goblet cell and secretory epithelial identity. Gene expression was quantified at 72 hours post-inoculation and normalized to *ACTIN* using the $\Delta\Delta C_t$ method with PBS-treated organoids as the reference condition.

In the MSS patient line, ST-GFP exposure drove significant upregulation of *TNF* ($**p < 0.01$ vs PBS; $***p < 0.001$ vs EcN-GFP), *LCN2* ($****p < 0.0001$ vs PBS; $***p < 0.001$ vs EcN-GFP), and *PDK4* ($*p < 0.05$ vs PBS), alongside significant induction of *SNAIL* ($**p < 0.01$ vs PBS and vs EcN-GFP) and *MUC2* ($*p < 0.05$ vs PBS and vs EcN-GFP; Figure 2.5A). *VIM* and *LGR5* trended toward change but did not reach significance in the MSS line. EcN-GFP produced modest and largely non-significant changes relative to PBS across all genes, confirming that the transcriptional response is ST-specific rather than a generic bacterial exposure effect.

Histological analysis using H&E and lipopolysaccharide (LPS) immunohistochemistry (IHC) on organoid sections provided complementary evidence of differential strain-organoid interaction. H&E staining revealed disrupted epithelial architecture and increased nuclear pleomorphism in ST-GFP-treated organoids relative to PBS and EcN-GFP controls, while LPS IHC demonstrated significantly elevated LPS-positive area in ST-GFP organoids compared to EcN-GFP ($**p < 0.01$) and PBS ($*p < 0.05$), with LPS puncta distributed intracellularly and

along the organoid luminal and basal surfaces (Figure 2.5B–C).

CellTiter-Glo viability assays at 72 hours showed no significant differences in bulk organoid viability between PBS, EcN-GFP, and ST-GFP conditions for either CRC or NC organoids at the inoculation doses employed (Figure 2.5D), indicating that the transcriptional and morphological changes observed in ST-GFP-treated organoids occur in the absence of overt cytotoxicity at the population level. Organoid area growth trajectories over 72 hours showed a significant difference between conditions ($**p < 0.01$), with PBS-treated organoids expanding to approximately $6\times$ their initial area on average, while EcN-GFP and ST-GFP organoids showed attenuated growth (fold changes of approximately 1.2 and 1.4 respectively; Figure 2.5E). This growth attenuation in bacterial co-culture conditions occurs without corresponding viability loss, suggesting cytostatic rather than cytocidal effects of colonization at the doses tested.

In the MSI line, the transcriptional profile showed partially conserved and partially divergent patterns relative to the MSS line (Figure 2.11A). *TNF* was significantly upregulated by ST-GFP in both lines, and *LCN2* and *PDK4* remained significantly elevated in the MSI line as well. However, the EMT program differed strikingly: *VIM* was significantly downregulated by ST-GFP in the MSI line ($**p < 0.01$), in contrast to the non-significant trend toward upregulation in the MSS line, while *LGR5* was significantly upregulated in the MSI line ($*p < 0.05$) but not the MSS line. These patient-specific transcriptional divergences are quantified formally in the cross-patient conservation analysis described in the following subsection.

2.2.6 Cross-patient conservation of transcriptional responses reveals a conserved inflammatory core and patient-specific EMT and metabolic programs

To characterize the inter-patient generalizability of the transcriptional response to ST colonization, we performed a cross-patient qPCR conservation analysis using $\log_2$ fold change (ST-GFP vs. PBS) as the effect size metric for each gene in the MSS and MSI lines (Figure 2.6C–D).

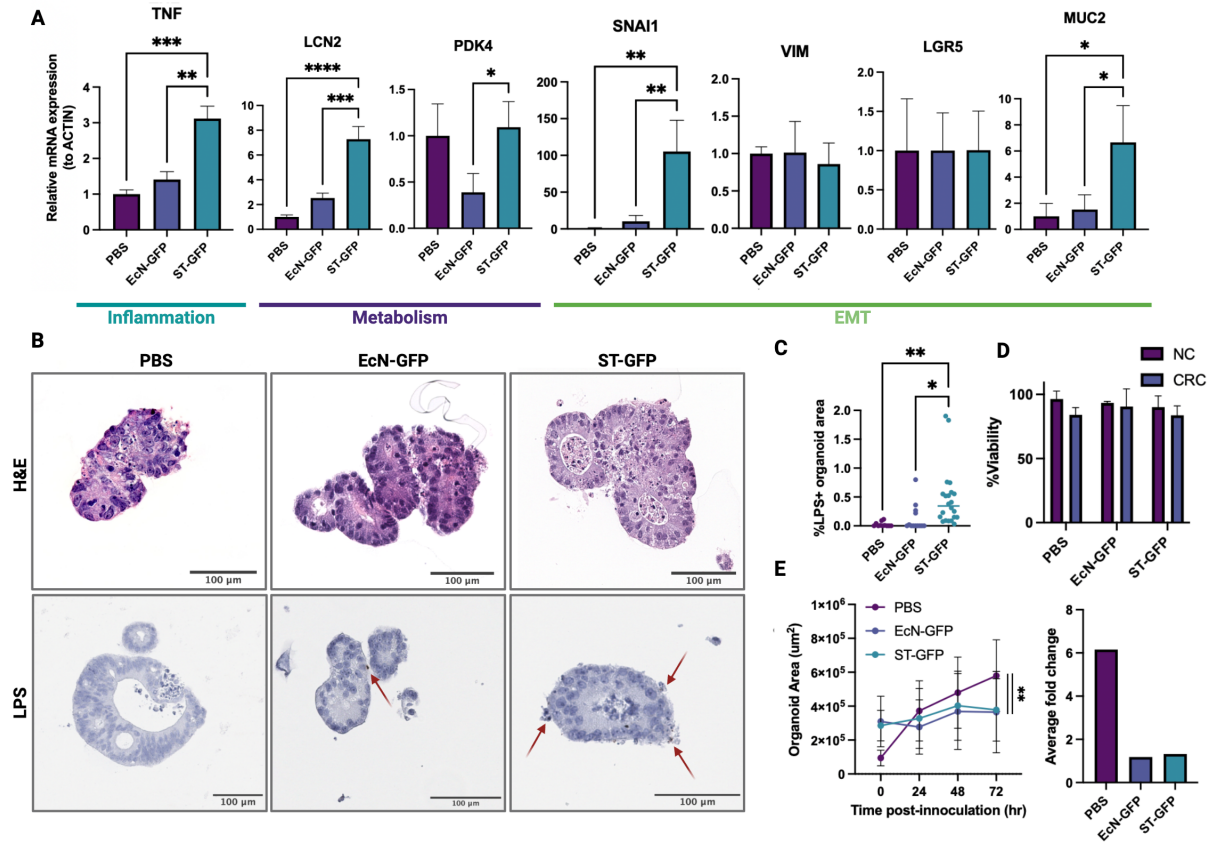


Figure 2.5. ST colonization drives strain-specific transcriptional remodeling and intracellular localization in CRC organoids. (A) Relative mRNA expression (normalized to ACTIN, calibrated to PBS) for a seven-gene panel in MSS CRC organoids at 72 hours with PBS, EcN-GFP, or ST-GFP. Gene categories: inflammation (*TNF*; teal), metabolism (*LCN2*, *PDK4*; purple), EMT (*SNAI1*, *VIM*, *LGR5*, *MUC2*; green). Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. (B) Representative H&E (top) and LPS IHC (bottom) images of MSS CRC organoid sections at 72 hours. Scale bars, 100 μ m. Red arrows indicate intracellular and luminal LPS puncta. (C) %LPS⁺ organoid area by condition. ST-GFP significantly elevated vs PBS (* $p < 0.05$) and EcN-GFP (** $p < 0.01$). (D) CellTiter-Glo %viability for NC and CRC organoids at 72 hours. No significant differences between conditions. (E) Organoid area (μ m²) over 72 hours (left) and average fold change (right). PBS-treated organoids expand significantly more than bacterial conditions (** $p < 0.01$).

In contrast to the morphological conservation analysis, qPCR responses showed substantially lower cross-patient correlation ($r = 0.268$, $p = 0.561$), indicating that transcriptional remodeling by ST is considerably more patient-specific than morphological remodeling (Figure 2.6C). By gene-level classification, *LCN2* and *TNF* were classified as “Conserved”. Both genes showed significant upregulation in both patient lines with directionally consistent effect sizes (Figure 2.6D). This conserved inflammatory core response, comprising innate immune activation (*TNF*) and epithelial stress signaling (*LCN2*), likely reflects a stereotyped host response to gram-negative bacterial invasion that is independent of CRC molecular subtype.

SNAIL showed high effect size in the MSS line ($\log_2FC \approx +7$) but was near-zero in the MSI line, classifying it as “Attenuated.” This attenuation is consistent with the MSI line’s different baseline epithelial architecture and reduced responsiveness to EMT-promoting stimuli. *MUC2* was classified as “Attenuated” in the same direction, with a strong MSS response that did not replicate in the MSI patient.

Strikingly, *VIM* and *PDK4* showed divergent responses between patient lines: *VIM* was significantly downregulated in the MSI line but non-significantly changed in the MSS line, while *PDK4* showed strong upregulation in the MSI line and only modest upregulation in the MSS line (Figure 2.6C–D). This divergence suggests that metabolic and mesenchymal reprogramming responses to ST colonization are highly patient-context-dependent and may reflect pre-existing transcriptional state differences between MSS and MSI tumors rather than fundamentally different cellular responses to bacteria. *LGR5* was classified as “Divergent” given significant upregulation in MSI but a non-significant direction change in MSS.

These conservation patterns collectively define a framework for interpreting bacterial therapeutic transcriptional responses in clinical translation: an inflammatory core (*TNF*, *LCN2*) can be expected to generalize across patients as a biomarker of bacterial activity, while EMT (*SNAIL*, *VIM*) and metabolic (*PDK4*) programs will require patient-specific interrogation.

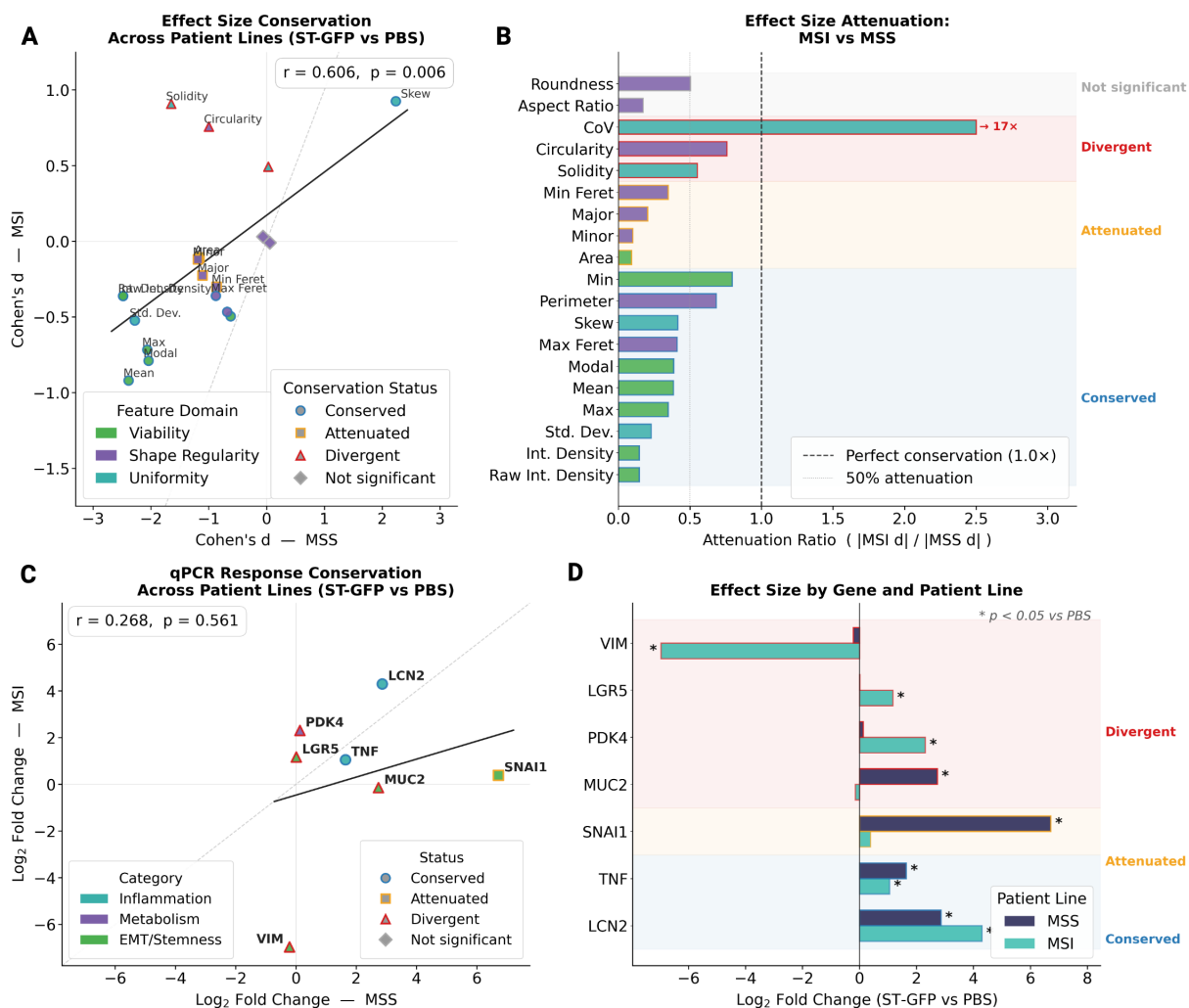


Figure 2.6. Cross-patient conservation of morphological and transcriptional responses to ST colonization (A) Scatter plot of Cohen's d effect sizes per morphometric feature: MSI line (y-axis) vs MSS line (x-axis) for ST-GFP vs PBS. Points colored by feature domain; shaped by conservation status. Pearson $r = 0.606$, $p = 0.006$. (B) Attenuation ratio bar chart ($|MSI d|/|MSS d|$) per feature, color-coded by conservation status (Conserved: blue; Attenuated: orange; Divergent: red; Not significant: grey). (C) Scatter plot of $\log_2$ fold change (ST-GFP vs PBS) per qPCR gene: MSI (y-axis) vs MSS (x-axis). Points colored by gene category. Pearson $r = 0.268$, $p = 0.561$. (D) Horizontal bar chart of $\log_2$ fold change by gene and patient line (MSS: dark navy; MSI: teal), grouped by conservation status. Asterisks indicate $p < 0.05$ vs PBS within each line.

2.2.7 ST colonization reduces secondary organoid-forming capacity in CRC cultures

To assess whether ST colonization impairs the regenerative and self-renewal capacity of CRC organoid cultures beyond the immediate co-culture window, we performed a secondary organoid-forming assay (OFA) in which primary CRC organoids were co-cultured with PBS, EcN-GFP, or ST-GFP for 72 hours, dissociated to single cells, washed extensively with antibiotic-containing media to eliminate residual bacteria, reseeded into fresh Matrigel, and cultured for five days before imaging and analysis (Figure 2.7A).

Brightfield imaging of secondary organoids revealed markedly reduced organoid density and size in the ST-GFP condition compared to PBS and EcN-GFP, with sparse, small-diameter organoid bodies predominating in the ST-GFP wells while PBS wells contained dense fields of well-formed organoids (Figure 2.7B). CellTiter-Glo viability assays on secondary organoids confirmed significantly reduced viability in ST-GFP wells relative to both PBS ($**p < 0.01$) and EcN-GFP ($**p < 0.01$), with ST-GFP viability averaging approximately 63% of control (Figure 2.7E). EcN-GFP secondary organoid viability was not significantly different from PBS, indicating that the reduction in regenerative capacity is ST-specific.

morphological analysis of secondary organoids by UMAP and feature heatmap demonstrated clear condition-level separation, with ST-GFP secondary organoids forming a distinct cluster in embedding space and showing uniformly reduced viability-domain feature values relative to PBS and EcN-GFP (Figure 2.7C–D). Violin plot comparisons confirmed that ST-GFP secondary organoids were significantly smaller (organoid size: $*p < 0.05$ vs PBS, $***p < 0.001$ vs EcN-GFP), displayed lower integrated density ($*p < 0.05$ vs PBS, $****p < 0.0001$ vs EcN-GFP), and had reduced Feret diameter ($*p < 0.05$ vs PBS, $***p < 0.001$ vs EcN-GFP), collectively indicating impaired organoid reconstitution after bacterial pre-treatment (Figure 2.7F).

In the MSI patient line OFA, viability trends were directionally preserved but effect sizes were attenuated: all three conditions showed similar viability at approximately 80–85%, with

no statistically significant differences between groups (Figure 2.12D). morphological analysis of MSI secondary organoids likewise showed condition-level UMAP separation that was less distinct than in the MSS line, and significant differences were found in organoid circularity (**** $p < 0.0001$ PBS vs ST-GFP; ** $p < 0.01$ PBS vs EcN-GFP) and solidity (* $p < 0.05$ PBS vs ST-GFP) but not in organoid size (Figure 2.12E). This pattern, in which shape regularity metrics differentiate conditions in the MSI OFA while absolute size and viability do not, is consistent with ST imposing structural reorganization on a cell population that is inherently more resistant to cytotoxic challenge.

Collectively, the OFA data demonstrate that ST colonization not only disrupts organoid architecture and transcription during co-culture but also has a lasting impact on the regenerative capacity of the surviving CRC cell population, an effect not observed with EcN. This finding is clinically significant in that it implies ST may target the cancer stem cell compartment (consistent with the morphological and LGR5 data) or otherwise deplete the self-renewing population responsible for tumor regrowth following therapy.

2.3 Methods

2.3.1 Bacterial strains and plasmid construction

Salmonella enterica serovar Typhimurium strain 1301 (ST) and *Escherichia coli* Nissle 1917 (EcN) were used as parental chassis for all experiments. GFP-expressing derivatives (ST-GFP, EcN-GFP) were generated by electroporating a constitutive GFP expression plasmid (pTET-GFP, ColE1 origin, ampicillin resistance) into electrocompetent cells prepared by successive washing in ice-cold 10% glycerol. Electroporation was performed at 1.8 kV, 25 μ F, 200 Ω in 1 mm cuvettes. Transformants were recovered in SOC medium for 1 hour at 37°C with shaking, plated on LB agar supplemented with 100 μ g/mL ampicillin, and screened for fluorescence by epifluorescence microscopy. Single colonies were propagated and GFP expression confirmed by flow cytometry and plate reader fluorescence before use in co-culture experiments. All

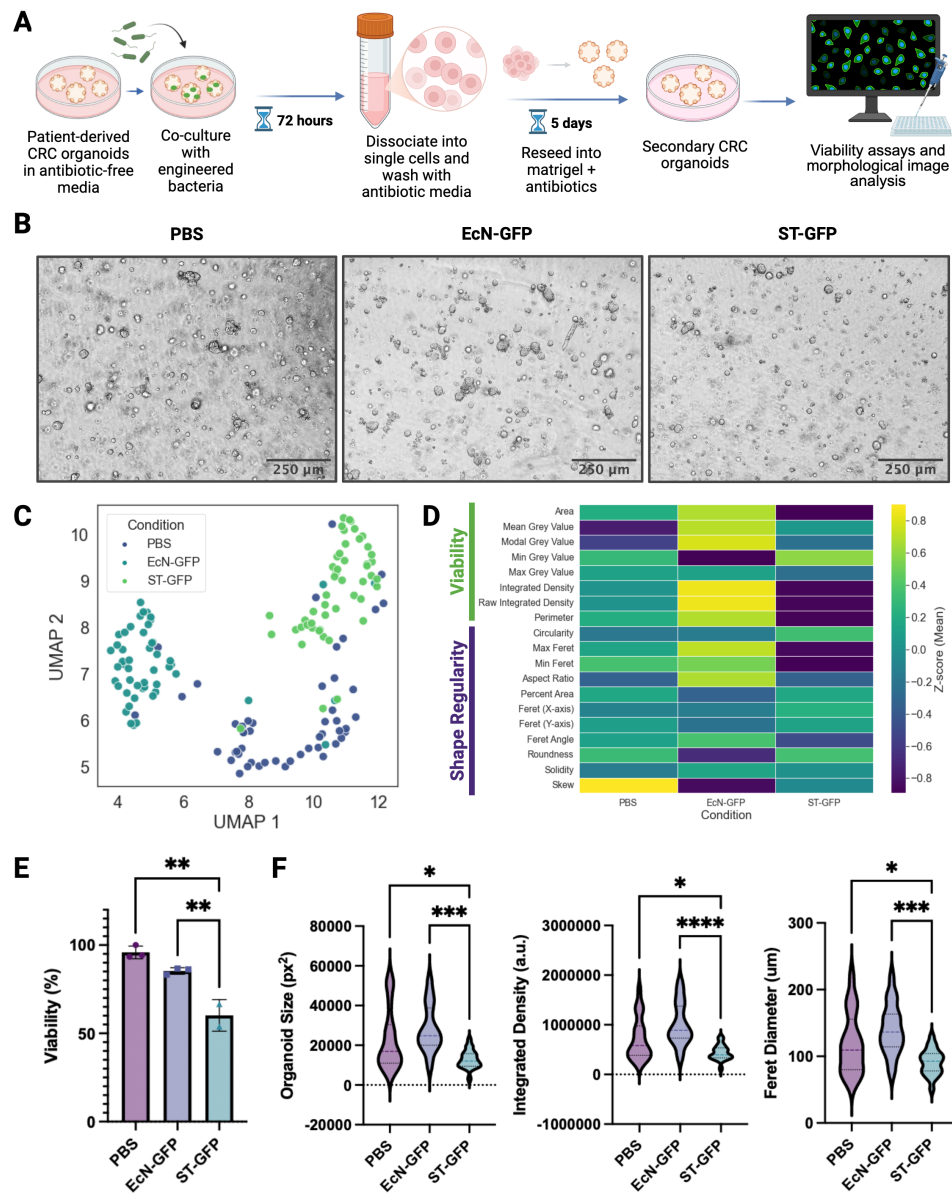


Figure 2.7. ST colonization reduces secondary organoid-forming capacity in CRC cultures. (A) Schematic of the secondary organoid-forming assay (OFA) workflow: primary CRC organoids co-cultured with PBS, EcN-GFP, or ST-GFP for 72 hours, dissociated to single cells, washed with antibiotic medium, reseeded into Matrigel, and cultured for 5 days. (B) Representative brightfield images of secondary MSS CRC organoids at day 5. Scale bars, 250 μm . ST-GFP wells show markedly reduced organoid density and size. (C) UMAP projection of secondary organoids colored by condition. (D) Heatmap of Z-score-normalized morphometric feature means by condition. (E) CellTiter-Glo %viability of secondary organoids. ST-GFP significantly reduced vs PBS (** $p < 0.01$) and EcN-GFP (** $p < 0.01$). (F) Violin plots of organoid size (px^2), integrated density (a.u.), and Feret diameter (μm). ST-GFP secondary organoids significantly smaller, less dense, and reduced Feret diameter vs both comparators (* $p < 0.05$ to **** $p < 0.0001$).

bacterial cultures for inoculation were grown overnight in LB broth at 37°C with 200 rpm shaking, subcultured 1:100 into fresh LB for 2–3 hours to mid-log phase, washed twice in sterile PBS, and resuspended to the desired optical density ($OD_{600} = 0.1$ unless otherwise specified) for inoculation.

2.3.2 Batch culture TME stressor screening

TME stressor screening was performed in 96-well microplates using a BioTek Synergy H1 multimode plate reader with simultaneous OD_{600} and GFP fluorescence (excitation 485 nm, emission 528 nm) monitoring at 15-minute intervals over 18 hours at 37°C with continuous orbital shaking. Four stressor categories were tested: (1) oxygen tension, achieved by sealing plates with BreatheEasy membrane and varying ambient oxygen concentration using a hypoxia chamber (0%, 10.5%, and 21.5% O_2); (2) pH, achieved by preparing LB medium buffered with 50 mM MES (pH 5.9, 6.88) or standard LB (pH 7.4) and adjusting with HCl/NaOH; (3) ROS, achieved by supplementing LB with hydrogen peroxide (H_2O_2) at 0, 0.2, or 0.6 mM immediately prior to inoculation; and (4) osmolarity, achieved by supplementing LB with NaCl to 0.3, 0.36, or 0.43 M. Each condition was tested in triplicate wells for both ST-GFP and EcN-GFP with a starting OD_{600} of 0.01. Growth rate (1/s) was calculated as the slope of the log-linear growth phase of the OD_{600} curve using a custom MATLAB fitting routine. Maximum GFP/OD was defined as the peak value of the GFP fluorescence: OD_{600} ratio across the time series. Coefficient of variance was computed for each metric across biological replicates within each condition. Statistical comparisons between stressor levels were performed using one-way ANOVA with Tukey's post-hoc test; significance thresholds are indicated as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

2.3.3 Patient-derived organoid establishment and maintenance

Patient-derived CRC organoids (MSS and MSI lines) and matched normal colon (NC) organoids were established from surgical resection specimens obtained from consented CRC

patients at [collaborating institution], under protocols approved by the [institutional review board]. Tumor and normal tissue specimens were processed within 4 hours of resection. Briefly, tissue fragments of approximately 1–2 mm³ were washed in cold PBS and subjected to enzymatic dissociation using 1 mg/mL collagenase IV (Sigma) supplemented with 10 μ M Y-27632 ROCK inhibitor in Advanced DMEM/F12 for 30–60 minutes at 37°C with gentle agitation. Dissociated cells were passed through a 100 μ m cell strainer, washed, and resuspended in cold Matrigel (Corning, growth factor reduced, phenol-red free) at a density of 500–1000 cells/ μ L. Domes of 40 μ L Matrigel were polymerized in pre-warmed 24-well plates at 37°C for 15 minutes, then overlaid with 500 μ L of complete organoid medium (Advanced DMEM/F12 supplemented with 10 mM HEPES, 2 mM GlutaMAX, 1 $\times$ B27, 1 mM N-acetyl cysteine, 50 ng/mL EGF, 100 ng/mL Noggin, 500 ng/mL R-Spondin-1, 50 ng/mL Wnt3a, 10 nM gastrin, 10 mM nicotinamide, 500 nM A83-01, 10 μ M SB202190, 100 μ g/mL Primocin). Organoids were maintained at 37°C in 5% CO₂ with medium changes every 2–3 days, passaged 1:3–1:4 every 7–10 days by mechanical disruption and re-embedding in fresh Matrigel. All co-culture experiments were performed at passages 3–8 to ensure phenotypic stability. Organoid identity was confirmed by morphological assessment and, for CRC lines, by targeted mutation panel sequencing to verify retention of patient-matched somatic variants.

2.3.4 PDO-bacteria co-culture protocol

For co-culture experiments, organoids were established in 96-well plates (Corning Ultra-Low Attachment, flat bottom) at a density of 50–100 organoids per well in 25 μ L Matrigel domes. Twenty-four hours prior to inoculation, organoid medium was replaced with antibiotic-free Advanced DMEM/F12 supplemented with 10 mM HEPES, 2 mM GlutaMAX, and 1 $\times$ B27 (co-culture medium) to clear residual antibiotic and allow bacterial survival. Bacteria were prepared as described above and diluted to OD₆₀₀ = 0.01 in co-culture medium immediately prior to inoculation. Each well received 200 μ L of bacterial suspension or PBS vehicle, yielding an approximate multiplicity of infection (MOI) of 10:1 (bacteria:organoid cells) based on

CFU calibration. Co-cultures were maintained at 37°C in 5% CO₂ without medium changes for the duration of the experiment (72 hours unless otherwise specified) to preserve bacterial colonization dynamics. For time-lapse experiments, plates were imaged at 0, 24, 48, and 72 hours post-inoculation on the BioTek Cytation 5 imaging plate reader.

2.3.5 Colony-forming unit (CFU) quantification

CFU quantification was performed at the designated endpoint (48 hours) by aspirating co-culture supernatant, washing wells twice with PBS, and lysing organoids in 200 μ L of 0.1% Triton X-100 in PBS for 10 minutes at room temperature with vigorous pipetting to disrupt Matrigel and organoid structures. Lysates were serially diluted in PBS and spot-plated (10 μ L per dilution) onto LB agar plates supplemented with 100 μ g/mL ampicillin to select for plasmid-retaining GFP-expressing bacteria. Plates were incubated overnight at 37°C and colonies counted manually or by automated image analysis. CFU per well was calculated from the dilution factor at which discrete colonies were countable (typically 10²–10⁴-fold dilution). Each condition was performed in at least three independent wells per experiment, and experiments were repeated across at least two independent organoid passages. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons or Kruskal-Wallis with Dunn's correction depending on normality assessment by Shapiro-Wilk test.

2.3.6 Fluorescence microscopy and GFP quantification

All fluorescence and brightfield imaging was performed on a BioTek Cytation 5 imaging multimode reader equipped with a 4 $\times$ or 10 $\times$ objective, GFP filter set (excitation 469/35 nm, emission 525/39 nm), and phase contrast module. For each well and time point, a 3 $\times$ 3 montage of overlapping fields was acquired and stitched using Gen5 software to produce a full-well composite image. GFP mean fluorescence intensity (MFI) was quantified from whole-well GFP channel images using a custom ImageJ macro that applied an Otsu threshold to the brightfield channel to generate an organoid area mask, then measured integrated GFP fluorescence within

that mask, normalized to total masked area (MFI/tumor area). Images used for morphological analysis were exported as uncompressed 16-bit TIFFs and analyzed separately as described below. All fluorescence quantification was performed on images acquired with identical hardware settings across all time points and experiments to ensure cross-condition comparability.

2.3.7 Morphological feature extraction and computational analysis pipeline

Brightfield images of individual organoids were segmented using a custom ImageJ/FIJI macro pipeline. Briefly, 8-bit converted brightfield images were background-subtracted using a rolling ball radius of 50 pixels, Gaussian blurred ($\sigma = 2$), and subjected to auto-thresholding using the Otsu method. Binary masks were processed by sequential hole-filling and watershed separation, and objects below a minimum size threshold of 500 px^2 were excluded to remove cell debris. The “Analyze Particles” function in ImageJ was used to extract 31 morphological features per organoid, including: area, mean/modal/min/max/integrated/raw integrated grey value, perimeter, centroid coordinates, center of mass coordinates, center of mass deviations (X, Y), major/minor axis lengths, angle, circularity, min/max Feret diameter, percent area, Feret X/Y/angle, aspect ratio, roundness, skew, solidity, standard deviation, and coefficient of variance.

Extracted features were exported as CSV files and imported into Python (v3.10) for downstream analysis. All features were standardized by Z-score normalization prior to multivariate analyses. Pairwise Spearman correlation matrices were computed using the `scipy.stats.spearmanr` package and visualized as heatmaps using `seaborn`. UMAP dimensionality reduction was performed using the `umap-learn` library (`n_neighbors = 15`, `min_dist = 0.1`, `n_components = 2`) on the full 31-feature standardized matrix. Linear regressions of individual features against per-organoid GFP MFI were performed using the `scipy.stats.linregress` package and R^2 values reported. ROC-AUC analysis was performed using the `sklearn.metrics.roc_auc_score` package with binary class labels (ST-GFP = 1, PBS + EcN-GFP = 0). Violin plots were generated in GraphPad Prism v9 and statistical comparisons performed by one-way ANOVA with Tukey’s

post-hoc test. All Python analysis code is available at [repository URL].

2.3.8 RNA extraction and quantitative RT-PCR

For qPCR analysis, co-culture wells were terminated by aspiration of medium, washing with cold PBS, and immediate addition of TRIzol reagent (Thermo Fisher) to lyse organoid and bacterial contents simultaneously. RNA was extracted per the manufacturer's protocol with an additional chloroform phase separation step, precipitated with isopropanol, washed with 75% ethanol, and resuspended in nuclease-free water. RNA concentration and purity were assessed by NanoDrop spectrophotometry ($A_{260}/A_{280} \geq 1.9$ for inclusion). Reverse transcription was performed using the SuperScript IV VILO Master Mix (Thermo Fisher) on 500 ng total RNA per reaction following the manufacturer's protocol. qPCR reactions were prepared with PowerUp SYBR Green Master Mix (Applied Biosystems) in 10 μ L reaction volumes containing 0.5 μ M of each primer and 2 μ L cDNA template (1:5 dilution). Thermocycling was performed on a QuantStudio 6 Flex system with the following conditions: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. All reactions included a melt curve analysis to confirm single-product amplification. Relative mRNA expression was calculated using the $\Delta\Delta C_t$ method with *ACTIN* (*ACTB*) as the reference gene and PBS-treated organoids as the calibrator condition. Gene-specific primer sequences are provided in Supplementary Table ???. Statistical comparisons were performed using one-way ANOVA with Tukey's multiple comparisons test.

2.3.9 Histology and LPS immunohistochemistry

For histological analysis, CRC organoids were co-cultured in 8-well chamber slides (Ibidi) under the standard co-culture protocol for 72 hours. Following co-culture, organoids were washed with PBS, fixed in 10% neutral buffered formalin for 24 hours at 4°C, and processed for paraffin embedding by standard methods (dehydration through graded ethanol, clearing in xylene, infiltration with paraffin at 60°C). Sections of 5 μ m thickness were cut from paraffin blocks, mounted on SuperFrost Plus slides, and baked at 60°C for 1 hour. Hematoxylin and

eosin (H&E) staining was performed using standard protocols on a Leica autostainer. For LPS IHC, sections were deparaffinized in xylene and rehydrated through graded ethanol, followed by heat-induced epitope retrieval in citrate buffer (pH 6.0) for 20 minutes at 95°C. Sections were blocked with 5% normal goat serum in PBS-T for 1 hour at room temperature, incubated with anti-LPS primary antibody (Abcam ab35654, 1:200 in blocking buffer) overnight at 4°C, washed with PBS-T, and incubated with HRP-conjugated secondary antibody (1:500) for 1 hour at room temperature. Signal was developed using DAB chromogen substrate (Vector Laboratories), sections were counterstained with hematoxylin, dehydrated, and mounted with Permount. Images were acquired on a Leica DM6 B upright microscope at 20× magnification. LPS-positive area was quantified using ImageJ by thresholding the DAB channel following color deconvolution (H-DAB vector) and measuring positive pixel fraction within a manually defined organoid boundary mask. Statistical comparisons across conditions used one-way ANOVA with Tukey's post-hoc test.

2.3.10 CellTiter-Glo 3D cell viability assay

Organoid viability was assessed using the CellTiter-Glo 3D Cell Viability Assay (Promega G9681) at the designated experimental endpoint. Wells were equilibrated to room temperature for 30 minutes, then CellTiter-Glo 3D reagent was added at a 1:1 volume ratio to the co-culture volume. Plates were shaken vigorously for 5 minutes to lyse cells and solubilize Matrigel, then incubated at room temperature for 25 minutes to stabilize luminescent signal. Luminescence was measured on a BioTek Synergy H1 plate reader with a 1-second integration time. Viability was normalized to the PBS control condition within each experiment and expressed as percent viability. Statistical comparisons were performed using one-way ANOVA with Tukey's post-hoc test.

2.3.11 Secondary organoid-forming assay

The secondary organoid-forming assay (OFA) was performed to assess the regenerative capacity of CRC organoid cultures following bacterial co-culture. Following 72 hours of co-culture under standard conditions, wells were washed twice with ice-cold Cell Recovery Solution (Corning) to depolymerize Matrigel, and organoids were collected by centrifugation at $400\times g$ for 5 minutes at 4°C . Organoid pellets were resuspended in TrypLE Express (Thermo Fisher) supplemented with $10\ \mu\text{M}$ Y-27632 and incubated at 37°C for 10 minutes with gentle pipetting to achieve single-cell dissociation. Dissociation was quenched with 10 volumes of cold complete organoid medium supplemented with $100\ \mu\text{g/mL}$ Primocin, and cells were centrifuged at $400\times g$ for 5 minutes. Cells were then resuspended in complete organoid medium supplemented with $200\ \mu\text{g/mL}$ Primocin and $50\ \mu\text{g/mL}$ gentamicin and incubated at 37°C for 2 hours with gentle rotation to eliminate residual extracellular bacteria. Following antibiotic treatment, cells were pelleted, washed with PBS, counted by hemocytometer with trypan blue exclusion, and reseeded at 1000 viable cells per $25\ \mu\text{L}$ Matrigel dome in 96-well plates under standard organoid culture conditions. Secondary organoids were cultured for five days before imaging on the Cytation 5 and CellTiter-Glo viability assessment. Morphological analysis of secondary organoids was performed using the same pipeline described above.

2.3.12 Statistical analysis

All statistical analyses were performed in GraphPad Prism v9 or Python (v3.10) as specified in the relevant subsections above. Normality of continuous variables was assessed by Shapiro-Wilk test; parametric (one-way ANOVA) or non-parametric (Kruskal-Wallis) tests were selected accordingly. Post-hoc multiple comparisons correction was applied using Tukey's Honest Significant Difference (HSD) for ANOVA or Dunn's test for Kruskal-Wallis. Significance thresholds are denoted as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ throughout all figures and text. For cross-patient conservation analyses, Pearson correlation coefficients

between MSS and MSI effect sizes were calculated in Python using `scipy.stats.pearsonr`; Cohen's d was computed as the mean difference divided by the pooled standard deviation. ROC-AUC values were calculated using `sklearn.metrics.roc_auc_score`. For attenuation ratio classification, features were assigned to "Conserved" (ratio 0.5–1.0 $\times$), "Attenuated" (ratio <0.5 $\times$), "Divergent" (ratio >1.0 $\times$ with sign reversal), or "Not significant" (MSI effect not reaching significance at $p < 0.05$) categories. All data are presented as mean $\pm$ SEM unless otherwise specified.

2.4 Acknowledgements

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Chapter 2 is under review for publication of the material as it may appear in *Communications Medicine*, 2026. The dissertation author was the primary investigator and author of this paper.

2.5 Supplementary Data

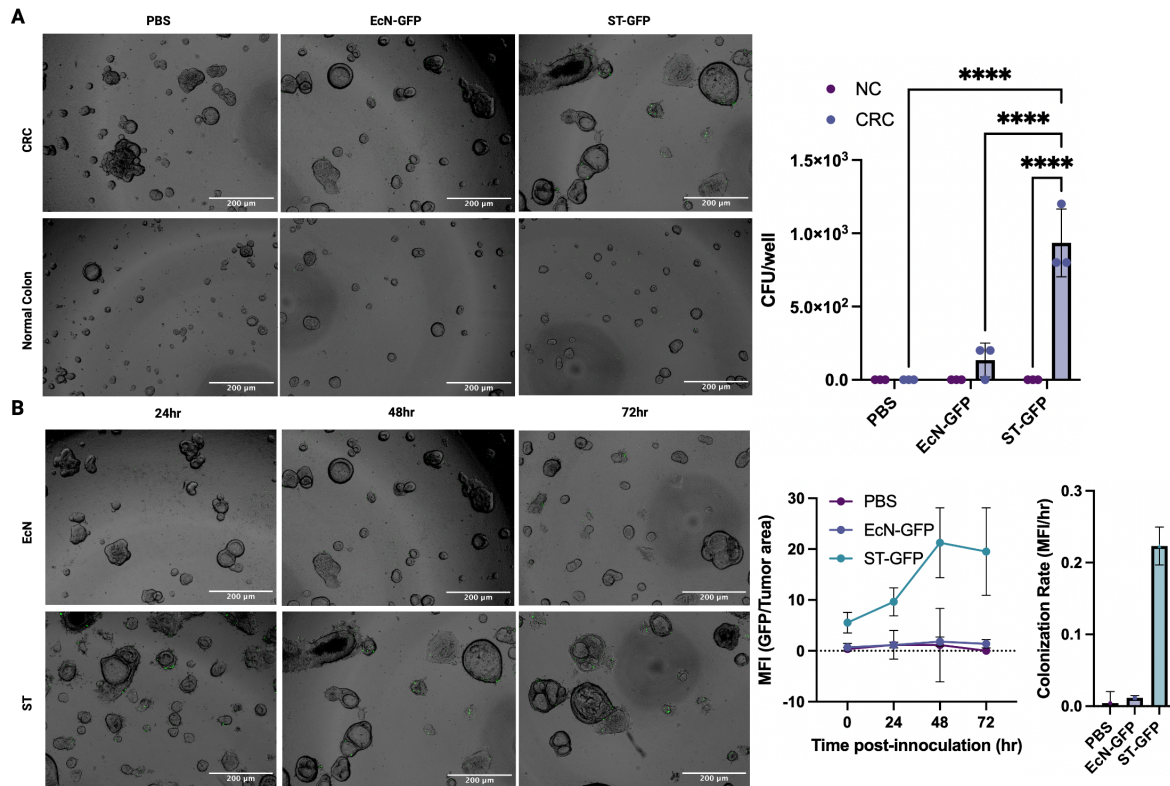


Figure 2.8. Supplementary Figure 1. MSI patient line: ST colonization efficiency and tumor specificity in PDO co-cultures (A) Representative brightfield and composite fluorescence images of MSI CRC (top) and matched normal colon (NC, bottom) organoids at 48 hours post-inoculation with PBS, EcN-GFP, or ST-GFP. Scale bars, 200 μ m. GFP signal is substantially reduced compared to the MSS primary line across all conditions. **(B)** CFU/well quantification from homogenized co-culture wells at 48 hours. ST-GFP achieves significantly higher CFU in CRC organoids compared to PBS and EcN-GFP (**** $p < 0.0001$), and CRC colonization significantly exceeds matched NC (**** $p < 0.0001$), confirming conserved tumor tropism in the MSI subtype. EcN-GFP colonization is negligible in both tissue types. **(C)** GFP MFI normalized to tumor organoid area over 72 hours post-inoculation in MSI CRC organoids for PBS (purple), EcN-GFP (blue), and ST-GFP (teal). ST-GFP shows a colonization kinetic profile directionally similar to the MSS line but with reduced peak MFI (~ 20 vs ~ 40 in MSS). Inset: colonization rate (MFI/hr) for each condition.

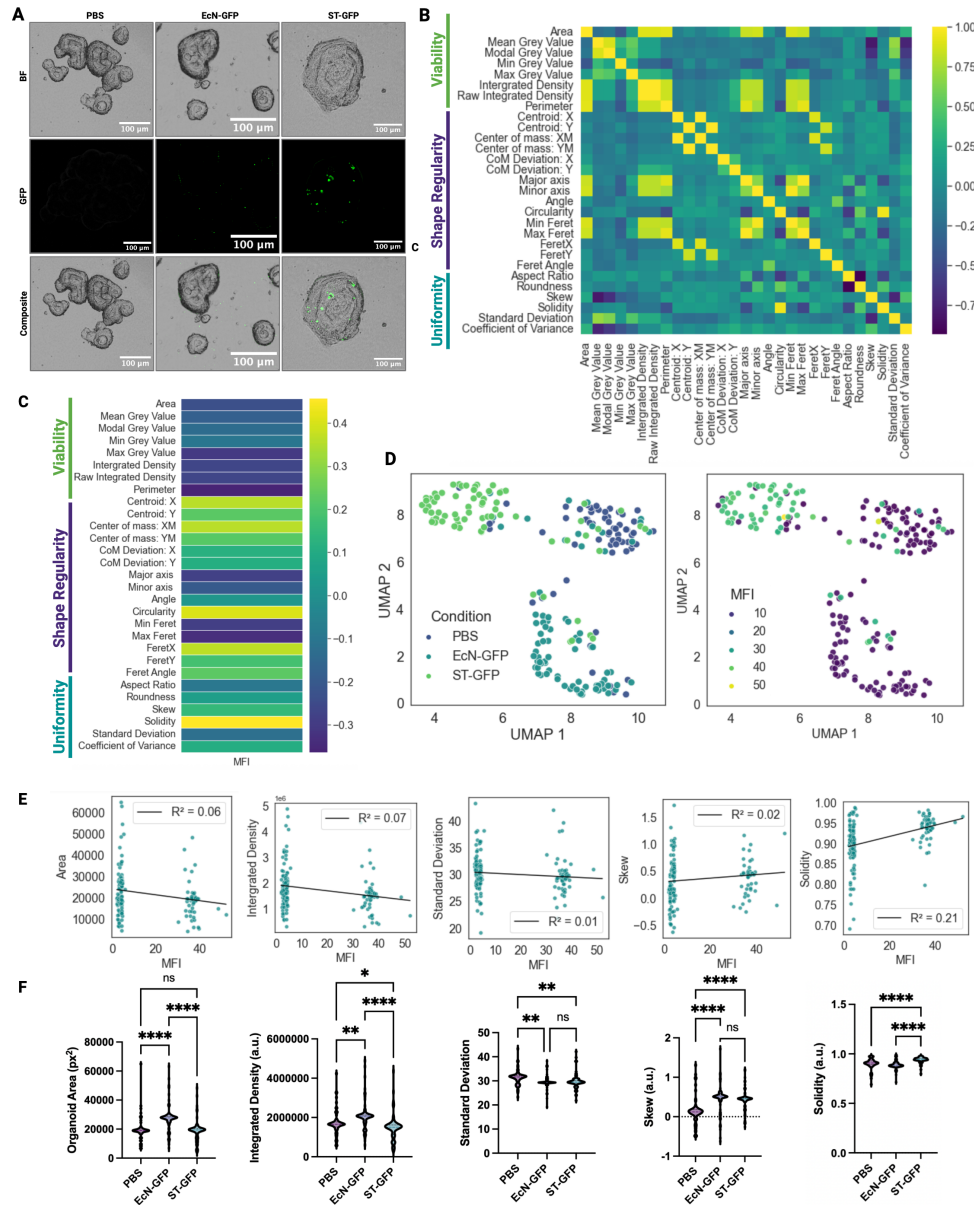


Figure 2.9. Supplementary Figure 2. MSI patient line: morphological analysis of PDO co-cultures (A) Representative brightfield (BF), GFP channel, and composite images of MSI CRC organoids at 48 hours for PBS, EcN-GFP, and ST-GFP conditions. Scale bars, 100 μm . (B) Pairwise Spearman correlation matrix of all morphological features computed from MSI organoid dataset. (C) Heatmap of Spearman correlations between each morphometric feature and GFP MFI in the MSI organoid dataset. (D) UMAP projection of all MSI organoids colored by condition (left) and continuous GFP MFI (right). (E) Linear regression of representative morphological features against GFP MFI in the MSI dataset. R^2 values shown. (F) Violin plots of representative morphological features by condition in the MSI dataset. Significant differences between conditions are retained for most features despite reduced effect sizes.

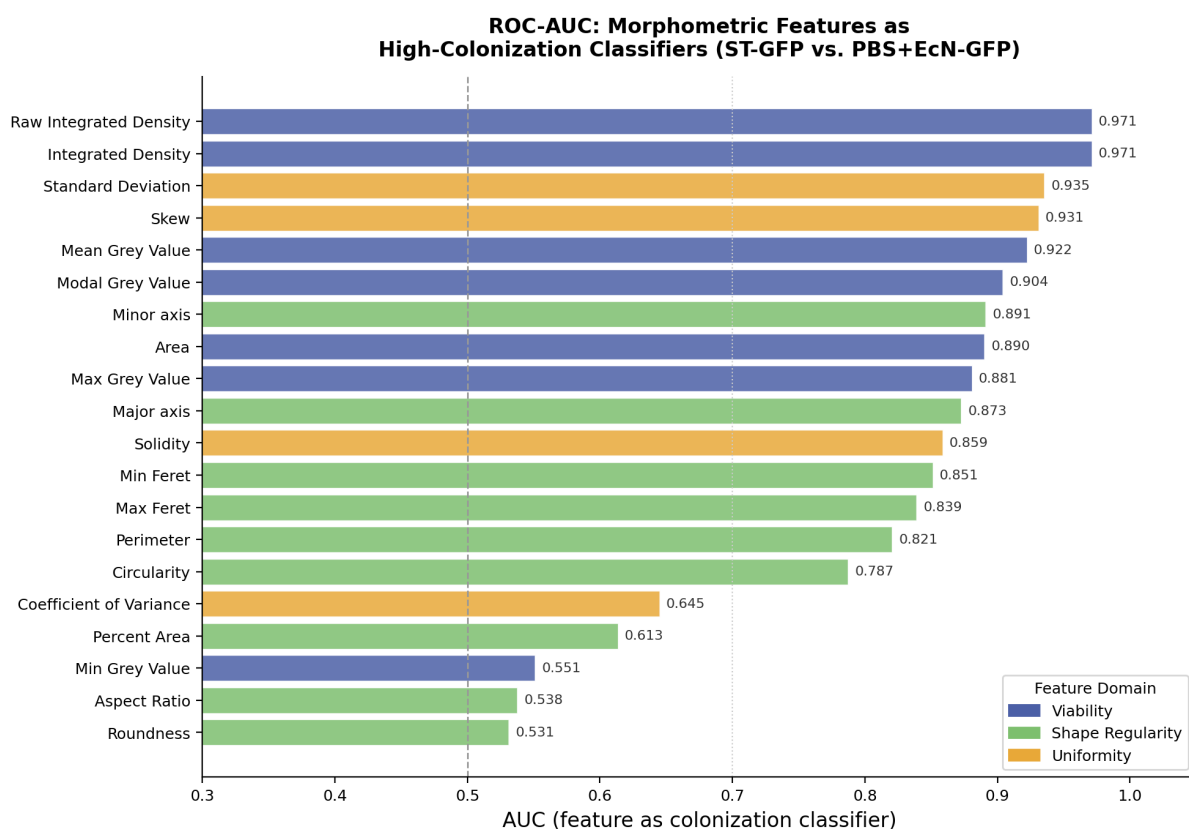


Figure 2.10. Supplementary Figure 3. ROC-AUC: morphological features as high-colonization classifiers ROC-AUC values for all morphological features as binary classifiers of high-colonization state (ST-GFP vs. PBS + EcN-GFP) in the MSS patient line. Features color-coded by domain: viability (blue), shape regularity (green), uniformity (orange). Dashed lines indicate AUC = 0.5 (chance) and AUC = 0.7 (acceptable). Raw integrated density and integrated density achieve AUC = 0.971 each, followed by standard deviation (0.935), skew (0.931), mean grey value (0.922). The discriminatory power of integrated density provides the methodological basis for morphological assessment of the untagged ST-pLux-Collagenase strain in Aim 3.

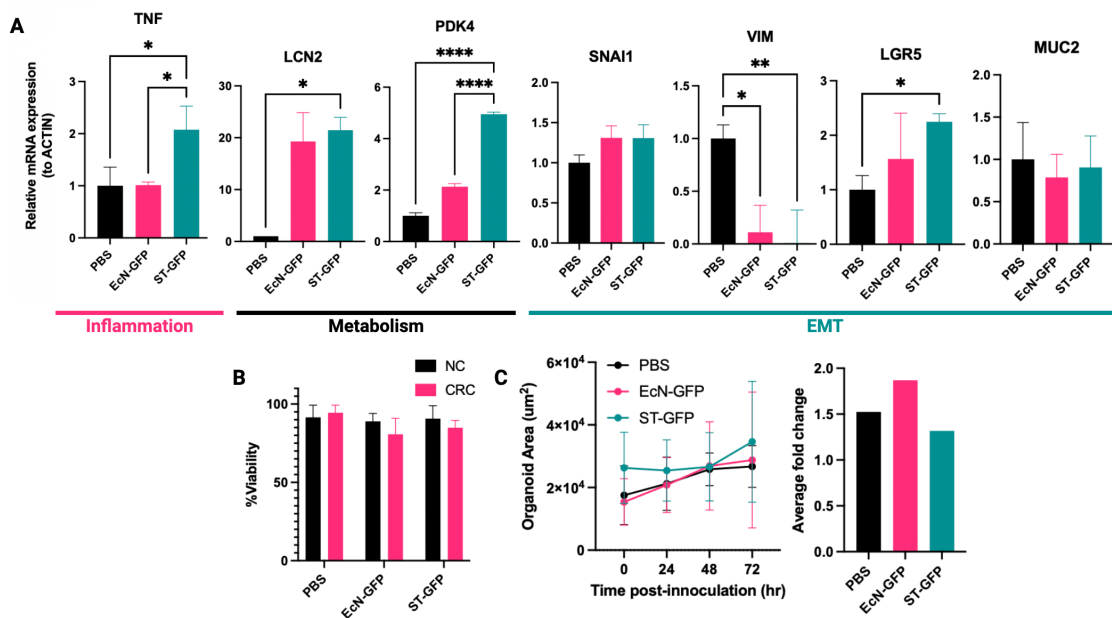


Figure 2.11. Supplementary Figure 4. MSI patient line: transcriptional response and organoid growth dynamics. (A) Relative mRNA expression (normalized to ACTIN, calibrated to PBS) for the seven-gene qPCR panel (TNF, LCN2, PDK4, SNAI1, VIM, LGR5, MUC2) in MSI CRC organoids at 72 hours post-inoculation with PBS, EcN-GFP, or ST-GFP. ST-GFP significantly upregulates TNF ($*p < 0.05$), LCN2 ($*p < 0.05$), and PDK4 ($****p < 0.0001$), and significantly downregulates VIM ($*p < 0.05$, $**p < 0.01$) and upregulates LGR5 ($*p < 0.05$) relative to PBS, in contrast to the MSS line pattern. Gene categories are indicated by color-coded bars below the x-axis (teal: Inflammation; black: Metabolism; teal: EMT). (B) CellTiter-Glo viability (% vs PBS) in MSI CRC and matched NC organoids at 72 hours. No significant differences in viability are observed between conditions in either tissue type, consistent with the MSS line pattern. (C) Organoid area (μm^2) time course (left) and average fold change from 0 to 72 hours (right) for PBS, EcN-GFP, and ST-GFP in MSI CRC organoids. All three conditions show similar growth trajectories with fold changes of 1.3–1.8 $\times$, contrasting with the significant PBS-driven growth advantage in the MSS line, consistent with generally attenuated organoid growth dynamics in the MSI patient line.

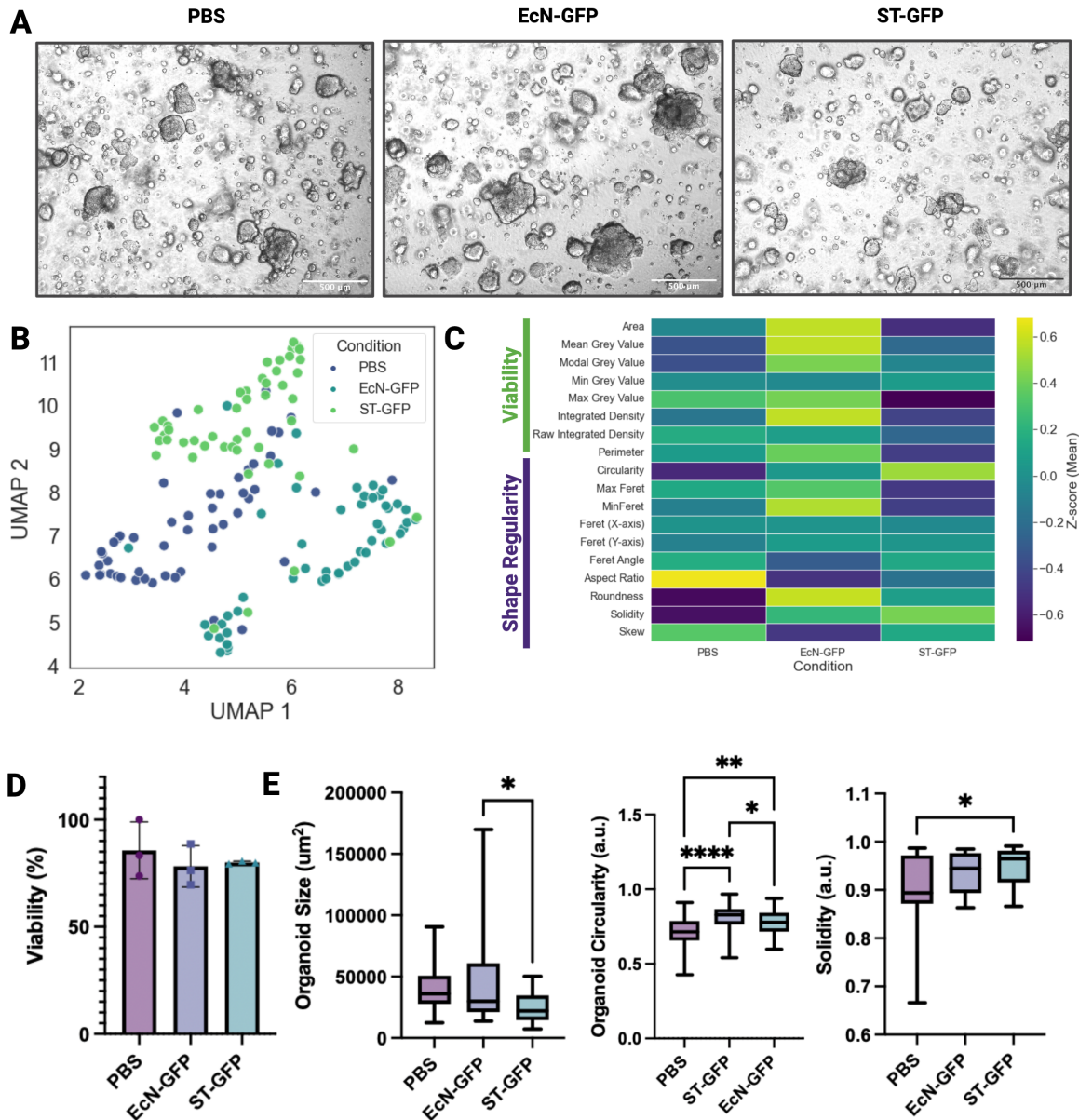


Figure 2.12. Supplementary Figure 5. MSI patient line: secondary organoid-forming assay. (A) Representative brightfield images of MSI secondary CRC organoids at day 5 of culture following PBS, EcN-GFP, or ST-GFP pre-treatment. Scale bars, 500 μ m. (B) UMAP projection of MSI secondary organoids colored by condition. (C) Feature heatmap of Z-score-normalized morphological feature means by condition in the MSI secondary organoid dataset. (D) CellTiter-Glo viability (%) of MSI secondary organoids by condition. (E) Morphological comparisons of MSI secondary organoids: organoid size (px²), organoid circularity (a.u.), and solidity (a.u.) by condition. Significant differences **** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$

Chapter 3

Evaluating Quorum-Regulated Collagenase as a Therapy Primer for Engineered Bacterial Therapeutics in Physiological Models of Colorectal Cancer

3.1 Summary

The preceding aims established that (i) quorum-regulated collagenase expression in *Salmonella typhimurium* 1301 enhances intratumoral bacterial penetration in HCT-116 spheroids, and (ii) ST achieves superior tumor-selective colonization relative to EcN in patient-derived colorectal cancer organoids, with colonization burden coupled to quantifiable and reproducible morphological signatures. Aim 3 brings these two threads together by evaluating the ST-pLux-Collagenase strain directly in patient-derived CRC organoids and in a syngeneic in vivo MC38 tumor model. Rather than framing the collagenase strain as a standalone cytotoxic agent, this aim advances a “therapy primer” concept: ST-pLux-Collagenase is deployed not to kill tumor cells directly, but to remodel the tumor ECM in a manner that sensitizes the tumor microenvironment to subsequent or co-administered therapeutic strategies, lowering the colonization threshold for a therapeutic partner strain and potentially reducing the dose of conventional therapy required to achieve efficacy. In patient-derived MSS and MSI organoids, ST-pLux-Collagenase achieved significantly higher CFU colonization than an ST-Empty vector control (**** $p < 0.0001$),

reduced organoid viability significantly below that of ST-Empty ($**p < 0.01$ MSS; $*p < 0.05$ MSI), and drove a distinct morphological signature detectable by fluorescence-independent brightfield analysis, with UMAP projections cleanly separating the collagenase condition from both PBS and ST-Empty. Transcriptional profiling by qPCR revealed collagenase-specific modulation of inflammatory, metabolic, and EMT gene programs in both patient lines, with notable attenuation of the ST-Empty inflammatory signature alongside unique collagenase-driven EMT changes. Critically, a co-inoculation experiment demonstrated that ST-pLux-Collagenase significantly enhances the colonization efficiency of a co-administered ST-GFP therapeutic proxy strain in CRC organoids from both patient lines ($*p < 0.05$, $***p < 0.001$), providing direct experimental evidence for the therapy primer concept. In a pilot in vivo study using the bilateral MC38 syngeneic mouse model, ST-pLux-Collagenase-treated tumors showed directional trends toward reduced intratumoral collagen content (trichrome fraction: 17.3% vs 41.8%), improved CD8⁺ T cell penetration depth, and elevated F4/80⁺ macrophage infiltration relative to ST-Control, consistent with ECM remodeling facilitating immune cell access to the tumor interior. These in vivo findings are interpreted as hypothesis-generating given the underpowered pilot cohort, but their directional alignment with the PDO mechanistic data provides encouraging translational support for the therapy primer strategy.

3.2 Results

3.2.1 ST-pLux-Collagenase achieves superior colonization and reduces viability in patient-derived CRC organoids across MSS and MSI subtypes

To evaluate the ST-pLux-Collagenase strain in a clinically representative tumor model, we co-cultured the untagged collagenase strain alongside an ST-Empty vector control (ST 1301 carrying the pLux backbone without collagenase insert) and a PBS vehicle control in matched CRC and normal colon (NC) organoids from both the MSS and MSI patient lines established in Aim 2. Critically, both ST-pLux-Collagenase and ST-Empty carry no fluorescent reporter

in this experimental design, making fluorescence-based colonization quantification unavailable and necessitating orthogonal readouts (CFU counts and CellTiter-Glo viability) for colonization and efficacy assessment. This experimental constraint was by design: the absence of a GFP tag removes any confounding contribution of reporter metabolic burden to observed phenotypes, and the use of CFU as the primary colonization metric provides a direct and unambiguous quantification of intratumoral bacterial burden that is independent of the morphological pipeline validated in Aim 2.

Brightfield imaging of MSS CRC organoids at 48 hours post-inoculation revealed qualitatively distinct phenotypes across conditions (Figure 3.1A, top block). PBS-treated organoids maintained well-defined, compact morphology with smooth outer boundaries. ST-Empty-treated organoids showed dense bacterial accumulation around and within organoid bodies, with visible disruption of the outer matrix consistent with ST colonization but without collagenase-mediated ECM remodeling. ST-pLux-Collagenase-treated organoids exhibited the most pronounced disruption: organoid boundaries were markedly less defined, the surrounding matrix was extensively fragmented, and individual organoids showed collapsed or dispersed architecture consistent with active collagenase-mediated ECM dissolution in the context of high bacterial burden. In matched NC organoids, all three conditions showed substantially reduced bacterial accumulation and preserved organoid morphology (Figure 3.1A, bottom block), consistent with the tumor-selective colonization phenotype established in Aim 2.

CFU quantification from homogenized MSS wells confirmed that ST-pLux-Collagenase achieved significantly higher intratumoral bacterial burden than both PBS (**** $p < 0.0001$) and ST-Empty (**** $p < 0.0001$), with ST-Empty itself significantly exceeding PBS (**** $p < 0.0001$; Figure 3.1C, left). NC organoids from both bacterial conditions showed near-zero CFU, confirming conservation of tumor-selective tropism in the collagenase strain. CellTiter-Glo viability assessment revealed that ST-pLux-Collagenase reduced MSS CRC organoid viability significantly below both No Bacteria (** $p < 0.01$) and ST-Empty (** $p < 0.01$) controls, with mean viability approximately 49% of the no-bacteria baseline, while ST-Empty viability was

not significantly different from no-bacteria controls at approximately 82% (Figure 3.1C, right). This pattern indicates that the collagenase strain exerts a cytotoxic or cytostatic effect on CRC organoids that exceeds the effect of bacterial colonization alone, consistent with collagenase-mediated physical disruption of the organoid ECM scaffold contributing to cell death beyond what ST infection itself produces.

In the MSI patient line, directional trends were conserved with attenuated magnitudes, consistent with the pattern established for all MSI readouts in Aim 2 (Figure 3.1C, lower panels). ST-pLux-Collagenase again significantly outperformed ST-Empty for CFU ($**** p < 0.0001$), and NC organoid colonization remained negligible for both strains. Viability in the MSI line showed a significant reduction in the ST-pLux-Collagenase condition relative to ST-Empty ($** p < 0.01$) and No Bacteria ($*p < 0.05$), though absolute viability values were less dramatically suppressed than in the MSS line, consistent with the MSI line's known resistance profile. Together, these colonization and viability data establish that ST-pLux-Collagenase achieves meaningfully superior tumor engagement relative to a vector-matched control across both CRC molecular subtypes, and that this superior engagement is associated with quantifiable tumor cell death at the organoid level.

3.2.2 ST-pLux-Collagenase drives a distinct fluorescence-independent morphological signature in MSS CRC organoids

Having validated the ROC-AUC performance of the morphological pipeline as an independent colonization classifier in Aim 2, we applied the same 31-feature brightfield analysis pipeline to the ST-pLux-Collagenase PDO dataset to characterize whether the untagged collagenase strain produces a morphological signature distinguishable from both PBS and ST-Empty without any fluorescence input. This analysis constitutes a direct functional application of the Aim 2 morphological methodology to the untagged therapeutic strain, the methodological scenario for which the $AUC = 0.971$ integrated density classifier was specifically developed as described in Aim 2 (Supplementary Figure 2).

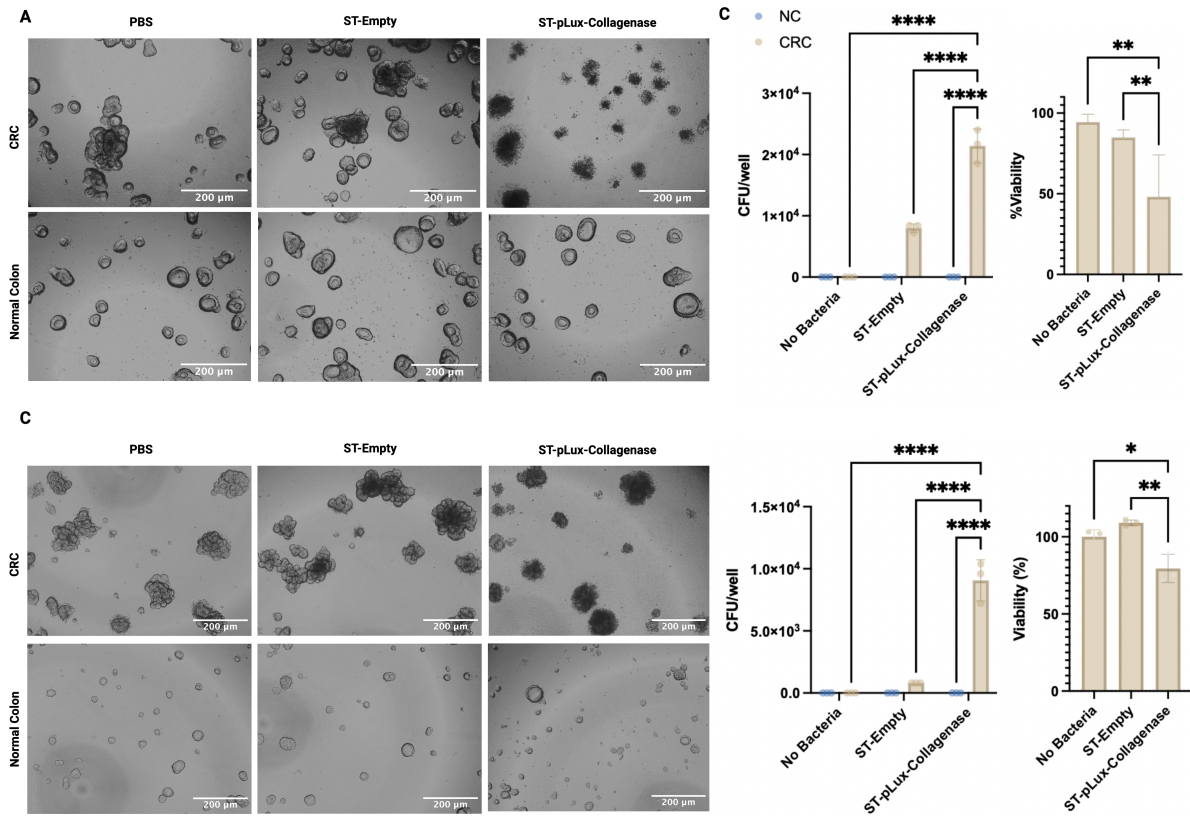


Figure 3.1. ST-pLux-Collagenase achieves superior colonization and reduces viability in patient-derived CRC organoids (A) Representative brightfield images of MSS CRC (top) and matched NC (bottom) organoids at 48 hours with PBS, ST-Empty, or ST-pLux-Collagenase. Scale bars, 200 μ m. (B) CFU/well from MSS wells at 48 hours. ST-pLux-Collagenase significantly exceeds No Bacteria (**** $p < 0.0001$) and ST-Empty (**** $p < 0.0001$); NC CFU near zero. Viability (%): ST-pLux-Collagenase significantly reduced vs No Bacteria (* $p < 0.01$) and ST-Empty (** $p < 0.01$). (C) Representative brightfield images of MSI CRC (top) and NC (bottom) organoids. Scale bars, 200 μ m. (D) CFU/well from MSI wells; directional pattern conserved at attenuated magnitude. ST-pLux-Collagenase significantly exceeds ST-Empty (**** $p < 0.0001$). Viability: ST-pLux-Collagenase significantly reduced vs ST-Empty (** $p < 0.01$) and No Bacteria (* $p < 0.05$).

Brightfield images of MSS CRC organoids confirm the visual correlates of the morphological stratification: PBS organoids are compact and well-bounded; ST-Empty organoids show intermediate disruption with bacterial mass accumulation around organoid bodies; ST-pLux-Collagenase organoids are extensively fragmented, with dark, collapsed cores and diffuse peripheral debris consistent with collagenase-mediated ECM dissolution (Figure 3.2A). UMAP dimensionality reduction of the full 31-feature morphological matrix produces a clear three-condition separation, with ST-pLux-Collagenase organoids forming a compact and well-isolated cluster in a distinct embedding region from both PBS and ST-Empty (Figure 3.2B). Notably, PBS and ST-Empty organoids show partial embedding overlap, while ST-pLux-Collagenase is fully separated, consistent with the collagenase strain producing a more extreme and more distinctive morphological state than bacterial colonization without ECM remodeling.

The feature heatmap of Z-score-normalized morphological means by condition confirms that viability-domain features (area, grey value statistics, integrated density) are most strongly differentiated across conditions, with ST-pLux-Collagenase showing markedly lower mean values across all viability features relative to PBS and ST-Empty (Figure 3.2C). Shape regularity features show intermediate differentiation, while uniformity features, particularly skew, show a striking pattern in which ST-pLux-Collagenase organoids have the highest mean skew, consistent with the asymmetric, collapsed morphology of extensively ECM-disrupted organoids. Violin plot comparisons confirm that both integrated density (**** $p < 0.0001$ for all pairwise comparisons) and skew (**** $p < 0.0001$ for all pairwise comparisons) are significantly different across all three conditions, with ST-pLux-Collagenase showing reduced integrated density and elevated skew relative to both comparators (Figure 3.2F). These results validate that the morphological pipeline, developed and calibrated on GFP-expressing ST in Aim 2, successfully detects and characterizes the colonization phenotype of the untagged ST-pLux-Collagenase strain using brightfield features alone, and further demonstrates that the collagenase-specific ECM disruption signature is morphologically distinguishable from non-collagenase bacterial colonization, providing a fluorescence-independent readout of both colonization and collagenase activity.

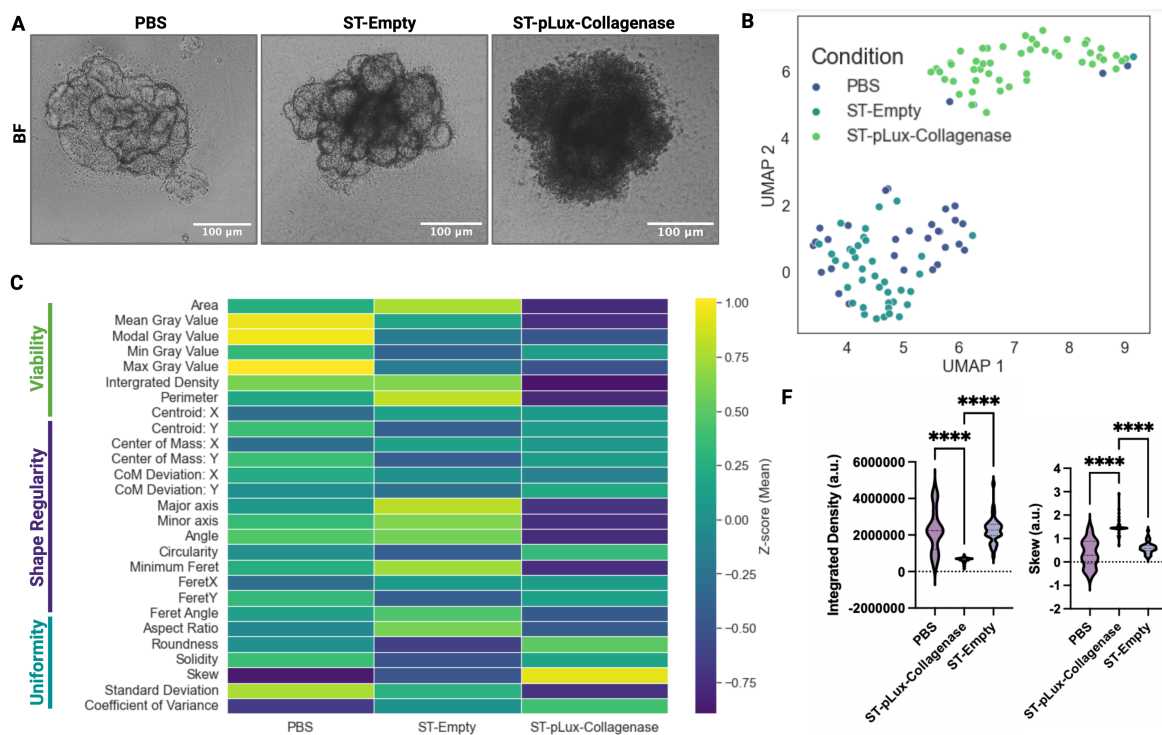


Figure 3.2. ST-pLux-Collagenase drives a distinct morphological signature in MSS CRC organoids. (A) Representative brightfield images of MSS CRC organoids at 48 hours with PBS, ST-Empty, or ST-pLux-Collagenase. Scale bars, 100 μm . (B) UMAP projection of MSS organoids colored by condition. ST-pLux-Collagenase organoids form a compact, fully isolated cluster. (C) Heatmap of Z-score-normalized morphometric feature means by condition, grouped by domain. Viability-domain features are most strongly differentiated; skew is highest in ST-pLux-Collagenase. (F) Violin plots of integrated density (a.u.) and skew (a.u.) by condition. All pairwise comparisons significant (**** $p < 0.0001$).

3.2.3 The collagenase morphological signature is conserved and further accentuated in the MSI patient line

Application of the morphological pipeline to the MSI CRC organoid dataset revealed a structurally analogous but phenotypically accentuated pattern relative to the MSS line (Figure 3.3). Brightfield images confirm that MSI organoids treated with ST-pLux-Collagenase show the most extensive architectural disruption of the three conditions, with large dark masses of fragmented organoid material and dissolved peripheral ECM (Figure 3.3A). UMAP projection produces an even cleaner three-condition separation in the MSI dataset than the MSS, with ST-pLux-Collagenase forming a completely isolated cluster with no overlap with PBS or ST-Empty (Figure 3.3B). This enhanced UMAP separation in the MSI line is notable given that the MSI line showed attenuated effect sizes for GFP-based colonization metrics in Aim 2. The morphological pipeline appears to be particularly sensitive to the physical ECM disruption produced by the collagenase strain, which may be especially visually dramatic in the MSI organoids due to their distinct baseline matrix architecture.

The feature heatmap confirms the same broad pattern of viability-domain feature suppression in ST-pLux-Collagenase organoids, with the additional notable feature that skew shows an even more extreme elevation in the MSI collagenase condition relative to PBS and ST-Empty than was observed in the MSS line (Figure 3.3C). Violin plots confirm highly significant differences in both integrated density (**** $p < 0.0001$ for all pairwise comparisons) and skew (**** $p < 0.0001$ for all pairwise comparisons; Figure 3.3D). The consistency of the morphological signature across both patient lines, despite the known biological differences between MSS and MSI CRC and the attenuated GFP-based colonization in the MSI line, supports the interpretation that collagenase-mediated ECM disruption produces a reproducible and patient-generalizable morphological phenotype that can serve as a robust readout of collagenase activity independent of fluorescent reporters.

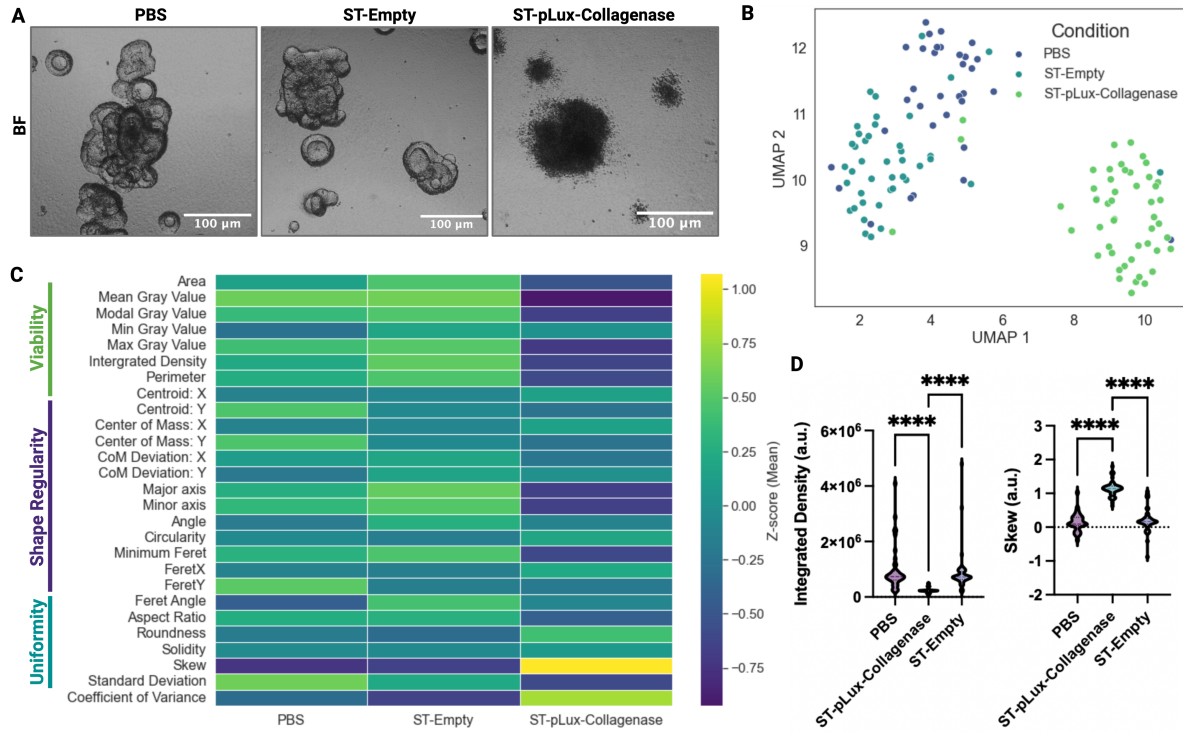


Figure 3.3. ST-pLux-Collagenase morphological signature is conserved and accentuated in the MSI patient line (A) Representative brightfield images of MSI CRC organoids at 48 hours with PBS, ST-Empty, or ST-pLux-Collagenase. Scale bars, 100 μ m. (B) UMAP projection of MSI organoids colored by condition. ST-pLux-Collagenase forms a completely isolated cluster with no overlap with PBS or ST-Empty. (C) Feature heatmap of Z-score-normalized morphometric means by condition. Skew elevation is more extreme than in MSS. (D) Violin plots of integrated density (a.u.) and skew (a.u.) by condition. All pairwise comparisons significant (**** $p < 0.0001$).

3.2.4 ST-pLux-Collagenase drives collagenase-specific transcriptional remodeling that is distinct from the ST-Empty inflammatory signature

To characterize the transcriptional consequences of collagenase-expressing versus control ST colonization in patient-derived CRC organoids, we performed qPCR analysis using the same seven-gene panel employed in Aim 2 (*TNF*, *LCN2*, *PDK4*, *SNAI1*, *VIM*, *LGR5*, *MUC2*) on organoids from both the MSI (Figure 3.4A) and MSS (Figure 3.4B) patient lines at 72 hours post-inoculation. Three conditions were compared: No Bacteria (reference), ST-Control (vector-only ST, equivalent to ST-Empty), and ST-pLux-Collagenase. This design allows decomposition of the transcriptional response into: (i) a general ST colonization effect (ST-Control vs. No Bacteria), and (ii) a collagenase-specific effect (ST-pLux-Collagenase vs. ST-Control).

In the MSI line (Figure 3.4A), ST-pLux-Collagenase significantly elevated both *TNF* ($*p < 0.05$ vs. No Bacteria; $**p < 0.01$ vs. ST-Control) and *LCN2* ($****p < 0.0001$ vs. No Bacteria; $****p < 0.0001$ vs. ST-Control) above the levels produced by ST-Control alone, indicating that collagenase expression amplifies the conserved inflammatory core response identified in Aim 2. *PDK4* was significantly elevated by ST-pLux-Collagenase relative to No Bacteria ($****p < 0.0001$) and ST-Control ($****p < 0.0001$), with ST-Control itself showing no significant *PDK4* induction in this line, suggesting that metabolic reprogramming toward glycolytic suppression is a collagenase-specific rather than ST-generic transcriptional effect in the MSI context. *SNAI1* was significantly upregulated by ST-pLux-Collagenase relative to ST-Control ($*p < 0.05$), indicating collagenase-driven EMT induction above the baseline ST response. Strikingly, *VIM* was significantly downregulated by ST-pLux-Collagenase relative to No Bacteria ($**p < 0.01$) and showed a trend toward reduction versus ST-Control ($**p < 0.001$), suggesting that collagenase-mediated ECM disruption paradoxically suppresses mesenchymal marker expression, possibly reflecting loss of the matrix-derived mechanical cues that sustain mesenchymal transcriptional programs in cancer cells [36]. *LGR5* and *MUC2* were not significantly modulated by either bacterial condition in the MSI line.

In the MSS line (Figure 3.4B), the transcriptional landscape showed a strikingly different pattern that underscores the patient-specificity of collagenase-driven transcriptional responses. *TNF* was significantly elevated by ST-Control ($***p < 0.001$ vs. No Bacteria) but significantly attenuated by ST-pLux-Collagenase relative to ST-Control, with ST-pLux-Collagenase values trending toward No Bacteria levels. This attenuation of the inflammatory response by the collagenase strain in the MSS line is the inverse of the amplification observed in the MSI line and may reflect a fundamental difference in how MSS CRC cells respond to the physical disruption of their ECM scaffold: in MSS organoids, where the ECM is particularly collagen-dense and structurally intact, collagenase-mediated matrix dissolution may reduce the mechanical and biochemical danger signals that drive *TNF* induction. *LCN2* was significantly elevated by ST-Control ($**p < 0.01$ vs. No Bacteria) but substantially suppressed by ST-pLux-Collagenase, reaching levels significantly below ST-Control. *SNAIL* showed a striking divergent pattern: significantly elevated by ST-Control ($***p < 0.001$) but profoundly suppressed by ST-pLux-Collagenase ($****p < 0.0001$ vs. ST-Control), indicating that collagenase-mediated ECM remodeling actively counteracts the EMT-promoting effect of ST colonization in the MSS context. *VIM* was significantly upregulated by ST-pLux-Collagenase relative to No Bacteria ($****p < 0.0001$) and ST-Control ($****p < 0.0001$), in contrast to the *VIM* suppression seen in the MSI line, further confirming the patient-line-specificity of mesenchymal transcriptional responses to collagenase. *LGR5* and *MUC2* were not significantly different across conditions in the MSS line.

The cross-patient pattern that emerges from these data is nuanced but interpretable within the therapy primer framework. The inflammatory core (*TNF*, *LCN2*) is modulated by collagenase in both patient lines, but in divergent directions (amplified in MSI and attenuated in MSS) consistent with the baseline transcriptional state of each subtype shaping the response to ECM disruption. The EMT program is consistently engaged by collagenase across both lines, though the specific markers that change differ between patients. Most importantly, the observation that ST-pLux-Collagenase produces a transcriptional profile that is distinct from ST-Control in both

lines confirms that the collagenase payload is biologically active at the tumor cell transcriptome level, not merely a physical ECM disruptor, and that its effects on gene expression are separable from the generic ST infection response.

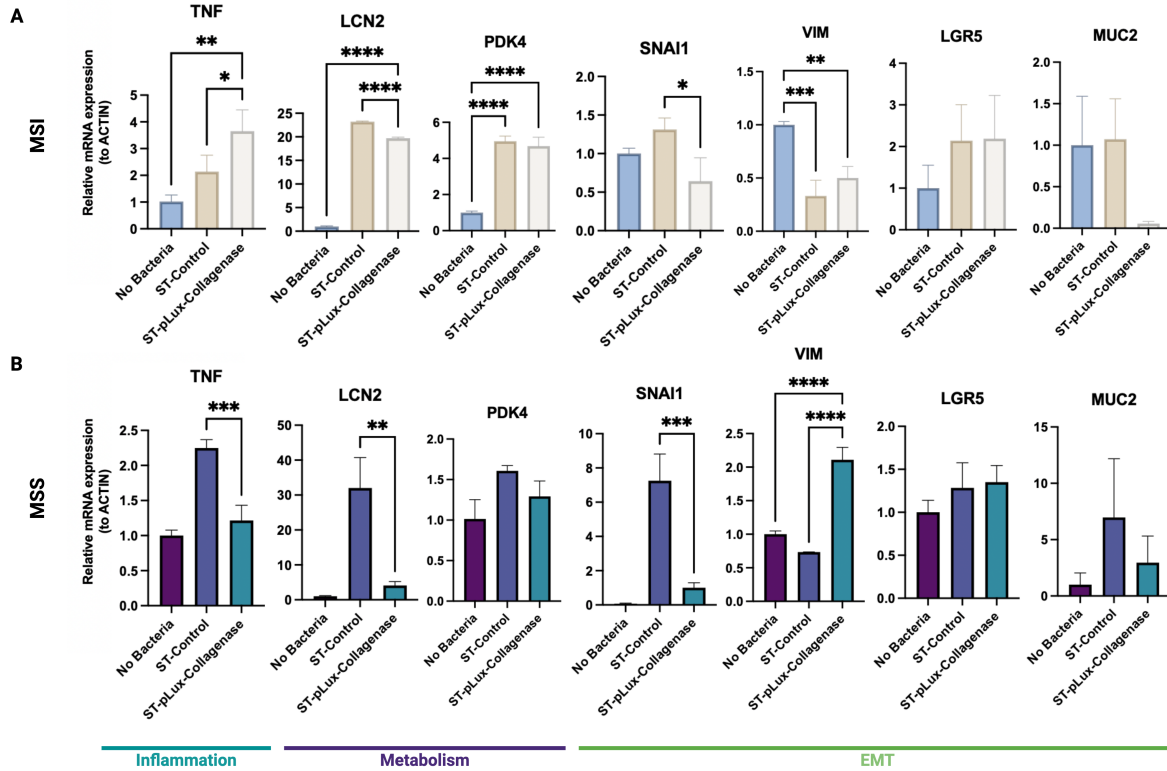


Figure 3.4. ST-pLux-Collagenase drives collagenase-specific transcriptional remodeling across MSI and MSS patient lines. (A) Relative mRNA expression (normalized to ACTIN, calibrated to No Bacteria) for the seven-gene qPCR panel in MSI CRC organoids at 72 hours with No Bacteria, ST-Control, and ST-pLux-Collagenase. Gene categories: inflammation (*TNF*; teal), metabolism (*LCN2*, *PDK4*; purple), EMT (*SNAI1*, *VIM*, *LGR5*, *MUC2*; green). ST-pLux-Collagenase amplifies *TNF* and *LCN2* above ST-Control and significantly suppresses *VIM*. **(B)** Same panel in MSS CRC organoids. ST-pLux-Collagenase attenuates the ST-Control-driven *TNF* and *LCN2* induction and profoundly suppresses *SNAI1* (**** $p < 0.0001$ vs ST-Control) while elevating *VIM* (**** $p < 0.0001$). The direction of collagenase effect on *TNF*, *VIM*, and *SNAI1* is opposite between patient lines.

3.2.5 ST-pLux-Collagenase functions as a therapy primer, significantly enhancing colonization of a co-administered therapeutic proxy strain

The therapy primer concept proposes that ST-pLux-Collagenase, by remodeling tumor ECM, creates a more permissive intratumoral environment that enhances the colonization efficiency of a co-administered therapeutic partner strain. To test this directly, we designed a co-inoculation experiment in which CRC organoids from both patient lines were simultaneously inoculated with two bacterial strains: ST-pLux-Collagenase (the “primer” strain, untagged) and ST-GFP (a GFP-expressing ST derivative serving as a proxy for a therapeutic partner strain). The GFP fluorescence of ST-GFP provides a direct, quantifiable readout of therapeutic proxy strain colonization, and the simultaneous presence of the collagenase primer allows real-time assessment of whether ECM remodeling by the collagenase strain enhances GFP strain penetration within the same co-culture well. The comparator condition was ST-Empty + ST-GFP (vector control primer + therapeutic proxy), enabling attribution of any differences in ST-GFP colonization specifically to the collagenase payload rather than to the general presence of a second ST strain.

In MSS CRC organoids, representative composite images at 2× and 10× magnification at 48 hours post-inoculation show markedly higher GFP signal in the ST-pLux-Collagenase + ST-GFP condition relative to ST-Empty + ST-GFP (Figure 3.5A). At 10×, GFP puncta in the collagenase co-inoculation condition are visible throughout the organoid body including the central core, while the ST-Empty + ST-GFP condition shows only sparse peripheral GFP signal, consistent with the collagenase strain opening ECM access routes that the GFP proxy strain exploits to achieve deeper intratumoral penetration. Time-lapse GFP MFI/tumor area over 72 hours confirms this divergence kinetically: the ST-pLux-Collagenase + ST-GFP condition shows elevated and sustained GFP MFI beginning at 24 hours and maintained through 72 hours, while ST-Empty + ST-GFP remains near-zero across the time course (Figure 3.5B). CFU quantification from dissociated wells at 72 hours confirmed that both the therapeutic proxy strain (ST-GFP) and

the primer strain (ST-pLux-Collagenase) achieved significantly higher CFU in CRC organoids in the collagenase co-inoculation condition relative to the control: therapeutic strain CFU was significantly elevated ($*p < 0.05$ vs. No Bacteria and vs. ST-Empty + ST-GFP), and facilitator strain CFU was significantly elevated ($***p < 0.001$ vs. No Bacteria; Figure 3.5C). NC organoid CFU remained negligible across all conditions, confirming conservation of tumor selectivity in the co-inoculation context.

In MSI CRC organoids, the co-inoculation experiment showed a directionally conserved pattern with attenuated absolute magnitudes (Figure 3.5D–F). The ST-pLux-Collagenase + ST-GFP condition showed higher GFP MFI at 24 hours relative to ST-Empty + ST-GFP, though the kinetic curve showed a declining trajectory from 24 to 72 hours in both conditions, consistent with the reduced sustained colonization in the MSI line (Figure 3.5E). CFU quantification confirmed significant elevation of therapeutic proxy strain CFU in the collagenase co-inoculation condition ($*p < 0.05$ vs. No Bacteria and vs. ST-Empty + ST-GFP; Figure 3.5F), establishing that the therapy primer effect generalizes to the MSI subtype despite attenuated magnitude.

These co-inoculation results provide direct experimental evidence for the therapy primer concept at the organoid level: the presence of the collagenase-expressing ST strain in the tumor environment significantly and specifically enhances the colonization efficiency of a co-administered therapeutic partner strain, above and beyond any enhancement attributable to co-colonization with a non-collagenase ST strain. Mechanistically, this effect is consistent with collagenase-mediated degradation of the pericellular collagen matrix creating physical access routes and reducing steric barriers that would otherwise limit therapeutic strain penetration into the organoid interior. Clinically, this finding supports a translational strategy in which ST-pLux-Collagenase is administered as a first-wave ECM primer to condition the tumor microenvironment prior to or concurrently with a therapeutic bacterial payload, potentially enabling lower doses of the therapeutic strain to achieve equivalent intratumoral coverage and reducing systemic exposure.

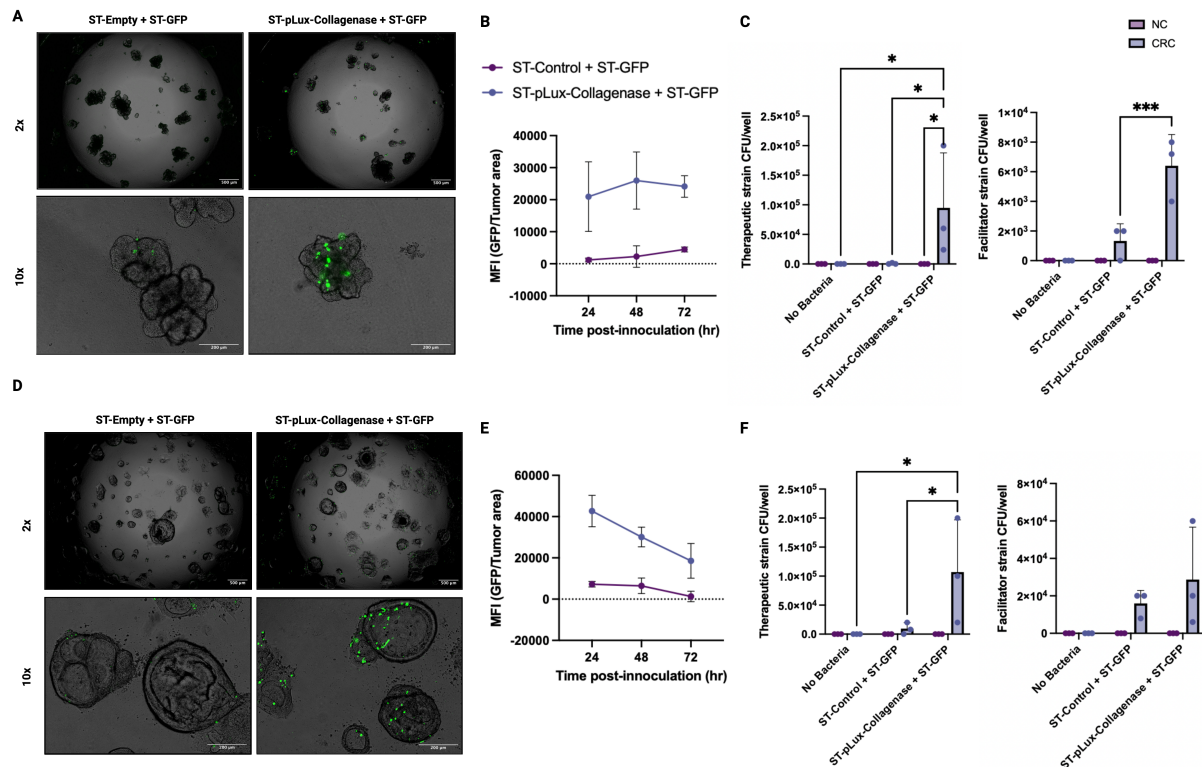


Figure 3.5. ST-pLux-Collagenase functions as a therapy primer, significantly enhancing colonization of co-administered therapeutic proxy strain. (A) Representative composite images of MSS CRC organoids at 48 hours with ST-Empty + ST-GFP (left) or ST-pLux-Collagenase + ST-GFP (right) at 2× (top) and 10× (bottom). Scale bars: 500 μ m (2×), 200 μ m (10×). (B) GFP MFI/tumor area over 72 hours in MSS organoids. ST-pLux-Collagenase co-inoculation sustains elevated MFI from 24 hours. (C) Left: therapeutic proxy strain (ST-GFP) CFU/well by condition; significantly higher in collagenase co-inoculation ($*p < 0.05$). Right: facilitator strain (ST-pLux-Collagenase) CFU/well; significantly elevated ($***p < 0.001$ vs No Bacteria). (D)–(F) Same layout for MSI organoids. GFP MFI elevated at 24 hours with declining kinetics. Therapeutic proxy CFU significantly elevated ($*p < 0.05$).

3.2.6 Pilot in vivo evaluation in the MC38 bilateral syngeneic mouse model reveals directional evidence for collagenase-mediated ECM remodeling and immune infiltration

To assess the in vivo translational potential of the ST-pLux-Collagenase therapy primer strategy, we conducted a pilot study using the bilateral MC38 syngeneic mouse model, in which C57BL/6 mice bearing bilateral subcutaneous MC38 colorectal tumors were treated with intratumoral injection of ST-Control or ST-pLux-Collagenase. Tumor tissues were harvested and processed for multiplexed immunofluorescence (mxIF) imaging and Masson's trichrome staining to assess intratumoral bacterial colonization (LPS signal), ECM collagen content (trichrome fraction), and immune cell penetration (CD8⁺ T cell and F4/80⁺ macrophage radial distribution). This pilot study was not powered for statistical significance (the cohort was limited in size due to an unplanned ulceration event in a subset of animals that necessitated early euthanasia, as described in the Experimental Design section) and all findings from this in vivo dataset are presented as exploratory and hypothesis-generating rather than confirmatory.

Representative mxIF whole-tumor images and Masson's trichrome sections at 2mm scale demonstrate qualitatively distinct tumor architectures between ST-Control and ST-pLux-Collagenase-treated animals (Figure 3.6A). Trichrome staining reveals that ST-Control tumors contain a dense, broadly distributed blue collagen matrix occupying a large fraction of the tumor cross-section, while ST-pLux-Collagenase-treated tumors show markedly reduced collagen staining with more fragmented, focal distribution and expanded regions of necrosis and cellular architecture. Computational stain segmentation of trichrome images (adipose excluded) quantifies this difference: mean collagen fraction in ST-Control tumors was 41.8%, compared to 17.3% in ST-pLux-Collagenase tumors, representing a 58.6% relative reduction in intratumoral collagen content (Figure 3.6B, left panel and collagen density maps). Necrotic fraction was correspondingly elevated in ST-pLux-Collagenase tumors (15.1% vs. 5.7% in ST-Control), consistent with collagenase-mediated ECM dissolution exposing previously matrix-encapsulated regions to degradation and cell death.

LPS immunofluorescence, used as a proxy for intratumoral bacterial colonization in mxIF, showed directional trends toward elevated LPS signal in ST-pLux-Collagenase-treated tumors relative to ST-Control (Figure 3.6D, Colonisation panel), though wide inter-animal variance in the underpowered cohort precludes statistical inference. This directional trend is consistent with the PDO data showing superior CFU in the collagenase condition, and with the therapy primer hypothesis that ECM remodeling by the collagenase strain enhances intratumoral bacterial persistence.

Co-localization analysis of LPS with F4/80 (macrophage marker) and CD8 (cytotoxic T lymphocyte marker) using spatial co-localization scoring revealed that ST-pLux-Collagenase-treated tumors showed higher LPS-F4/80 and LPS-CD8 co-localization scores in the regions of highest bacterial density (Figure 3.6C), suggesting that collagenase-mediated ECM disruption facilitates immune cell co-infiltration into bacterially colonized regions. Radial slope analysis of CD8⁺ T cell and F4/80⁺ macrophage signal as a function of distance from the tumor center provides a direct measure of immune cell penetration depth: a less negative (or positive) radial slope indicates that immune cells are distributed more uniformly throughout the tumor rather than being excluded from the interior (Figure 3.6D, right panels). ST-pLux-Collagenase-treated tumors showed directionally less negative CD8 radial slopes (mean $\approx +0.0001$ intensity/px) compared to ST-Control (mean ≈ -0.0004 intensity/px), and less negative F4/80 radial slopes (mean ≈ -0.00025 intensity/px vs. ≈ -0.00075 intensity/px in ST-Control). These directional trends, while not reaching statistical significance in the current cohort, are consistent with the mechanistic prediction that collagenase-mediated collagen reduction lowers the physical barrier to immune cell migration into the tumor core.

It is important to contextualize these in vivo findings honestly. The pilot cohort was underpowered due to an unplanned ulceration event that disproportionately affected one treatment arm and required early euthanasia of affected animals, reducing the effective sample size below what was needed for robust statistical comparison. The directional alignment of all four measured endpoints (increased colonization, reduced collagen, improved CD8 penetration, improved

macrophage infiltration) with the a priori mechanistic hypothesis is encouraging and provides a coherent translational narrative, but should not be interpreted as confirmatory evidence. A powered second cohort incorporating the imaging and analysis pipeline developed here is planned and will provide the definitive in vivo test of the therapy primer strategy. Nevertheless, the consistency between the in vivo directional trends and the mechanistic PDO data from the same strain provides meaningful convergent support for the hypothesis, and the imaging pipeline itself represents a validated analytical framework that will be immediately deployable in the planned follow-up study.

Colonization asymmetry in the ST-Control bilateral animal suggests hematogenous trafficking to the contralateral tumor

Spatial analysis of LPS signal across the two bilateral tumors in the single ST-Control animal and subsequent CFU plating of tissue homogenates revealed reversal of the anticipated colonization pattern: the directly injected tumor (4LL_LT) exhibited markedly lower LPS signal and reduced LPS-immune co-localization scores relative to the non-injected contralateral tumor (4LL_RT), which showed substantially higher LPS intensity and broader spatial distribution across the tissue section (Supplementary Figure 3.7a–b). This pattern is inconsistent with purely local bacterial growth at the injection site and instead suggests that a fraction of the injected population disseminated hematogenously from the injection site and established preferential colonization in the undisturbed contralateral tumor.

Cross-metric correlation analysis across all ST-Control and ST-pLux-Collagenase samples, presented as hypothesis-generating scatter plots given the sample sizes involved (Supplementary Figure 3.7c), provides additional context. In the colonization–T cell response, 4LL_LT occupies the highest CD8 signal position despite near-zero LPS signal, consistent with a residual immune response at the injection site in the absence of sustained bacterial colonization. In the ECM collagen versus CD8 penetration depth panel, 4LL_LT is the most extreme outlier in the dataset: it carries the highest collagen fraction of any sample (0.418) and the most negative

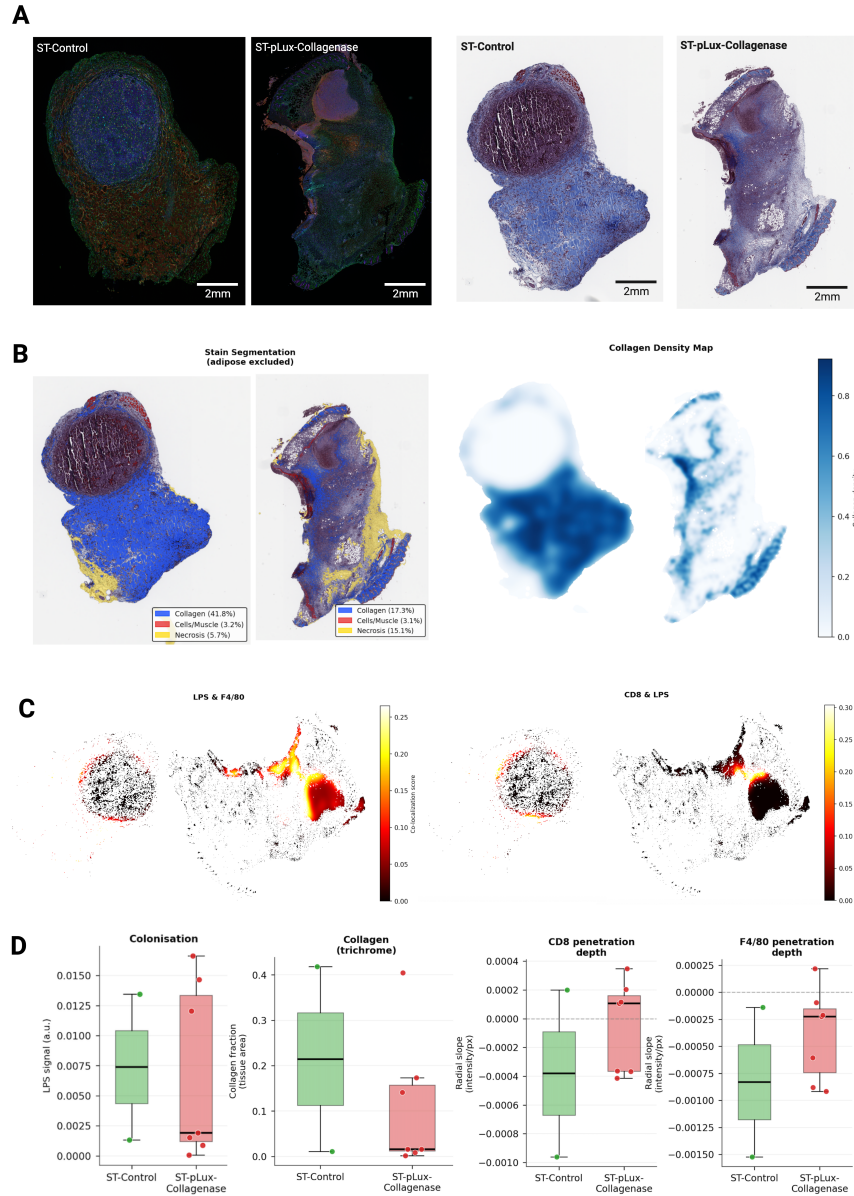


Figure 3.6. ECM remodeling and immune infiltration trends in subcutaneous MC38. (A) Representative mxIF whole-tumor images (ST-Control and ST-pLux-Collagenase) and matched Masson's trichrome sections at 2 mm scale. Trichrome staining shows dense collagen in ST-Control tumors and markedly reduced, fragmented collagen in ST-pLux-Collagenase tumors. (B) Left: computational stain segmentation (adipose excluded) showing collagen (blue), cells/muscle (red), and necrosis (yellow). Mean collagen fraction: 41.8% (ST-Control) vs 17.3% (ST-pLux-Collagenase). Right: spatially resolved collagen density maps. (C) Spatial co-localization heatmaps for LPS $\times$ F4/80 (left) and CD8 $\times$ LPS (right) for representative tumor sections. Higher co-localization scores in collagenase-treated tumors indicate greater immune cell co-infiltration into bacterially colonized regions. (D) Boxplots of four summary metrics by treatment group: colonization (LPS signal), collagen fraction (trichrome), CD8 penetration depth (radial slope), F4/80 penetration depth (radial slope). All trends directionally consistent with therapy primer hypothesis; no inferential statistics applied given underpowered cohort.

CD8 radial slope (-0.00097), indicating that immune infiltration is concentrated peripherally and excluded from the ECM-dense core (the canonical immune-excluded phenotype against which the therapy primer strategy is designed). This profile stands in qualitative contrast to the ST-pLux-Collagenase samples, which cluster at lower collagen fractions with CD8 slopes distributed around zero or positive, consistent with reduced ECM barrier and immune access to the tumor interior.

3.3 Methods

3.3.1 Bacterial strains

All experiments in this chapter used ST 1301 derivatives. ST-pLux-Collagenase is the quorum-regulated, lysis-deficient collagenase strain described in Aim 1 (ST-pLux-Collagenase), carrying no fluorescent reporter. ST-Empty is an isogenic strain carrying the pLux backbone plasmid with the collagenase insert replaced by a null stuffer sequence of equivalent length, preserving metabolic burden equivalence while eliminating collagenase activity. ST-GFP is the constitutive GFP-expressing ST 1301 derivative described in Aim 2, used as a therapeutic proxy strain in the co-inoculation experiment. All strains were prepared from overnight LB cultures as described in Aim 1 (Methods: Bacterial Culture Conditions), adjusted to $OD_{600} = 0.5$ in sterile $1 \times$ DPBS immediately prior to inoculation. For co-inoculation experiments, ST-pLux-Collagenase and ST-GFP were prepared as separate suspensions at $OD_{600} = 0.5$ and combined 1:1 immediately before addition to organoid wells, such that each strain contributed half the total inoculation volume.

3.3.2 Patient-derived organoid co-culture with collagenase strains

PDO establishment, maintenance, and co-culture were performed as described in Aim 2 (Methods: Patient-Derived Organoid Establishment and Maintenance; PDO-Bacteria Co-culture Protocol) using the MSS and MSI patient lines. For Aim 3 co-culture experiments,

the bacterial inoculation and washing protocol was identical to Aim 2, with the substitution of ST-pLux-Collagenase and ST-Empty in place of ST-GFP and EcN-GFP. Co-cultures were maintained for 48–72 hours depending on the assay endpoint. CFU quantification and CellTiter-Glo viability assays were performed exactly as described in Aim 2. For the co-inoculation experiment (Figure 3.5), both ST-pLux-Collagenase and ST-GFP were inoculated simultaneously at the same total bacterial input density, with each strain contributing 1 μL to a total 2 μL inoculation per well. Post-washing and gentamicin addition were performed identically to standard co-culture. For the co-inoculation CFU analysis, the two strains were distinguished by plating dissociated well lysates on selective agar: LB + kanamycin for ST-GFP (therapeutic proxy, kanamycin-resistant GFP plasmid), and LB + chloramphenicol for ST-pLux-Collagenase (collagenase plasmid, chloramphenicol-resistant), enabling independent quantification of each strain’s intratumoral CFU from the same well.

3.3.3 morphological analysis of collagenase PDO co-cultures

Brightfield morphological analysis of ST-pLux-Collagenase and ST-Empty PDO co-cultures was performed using the identical pipeline described in Aim 2 (Methods: morphological Feature Extraction and Computational Analysis Pipeline). Briefly, brightfield images were acquired on the BioTek Cytation 5 at $4\times$ magnification, segmented using the custom ImageJ/FIJI macro, and 31 morphological features were extracted per organoid. All downstream Python analyses (UMAP, Z-score heatmaps, violin plots) were performed with identical parameters to the Aim 2 pipeline to ensure cross-experiment comparability. In this experimental context, the morphological pipeline functions as a fluorescence-independent readout of collagenase colonization state, applying the integrated density and skew classifiers validated by ROC-AUC analysis in Aim 2 to the untagged collagenase strain dataset. Statistical comparisons of morphological features across conditions were performed using one-way ANOVA with Tukey’s post-hoc test.

3.3.4 RNA extraction and quantitative RT-PCR

qPCR analysis for Aim 3 was performed using the identical protocol described in Aim 2 (Methods: RNA Extraction and Quantitative RT-PCR), using the same seven-gene panel (TNF, LCN2, PDK4, SNAI1, VIM, LGR5, MUC2) normalized to ACTIN with No Bacteria as the calibrator condition. Three conditions were compared: No Bacteria, ST-Control, and ST-pLux-Collagenase. RNA extraction, cDNA synthesis, and qPCR thermocycling conditions were identical across Aims 2 and 3 to ensure cross-aim comparability of transcriptional readouts.

3.3.5 MC38 syngeneic mouse model and intratumoral bacterial injection

All animal experiments were conducted under IACUC-approved protocols at [institution]. Female C57BL/6 mice (6–8 weeks, Taconic Biosciences) were acclimated for one week prior to tumor cell injection. MC38 murine colorectal adenocarcinoma cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. For bilateral tumor establishment, mice were injected subcutaneously in both flanks with 5×10^5 MC38 cells in 100 μ L sterile PBS. Tumors were monitored by caliper measurement (volume = length $\times$ width $\times$ height) twice weekly. When bilateral tumors reached approximately 150 mm³, mice were randomized across treatment groups. Bacterial suspensions were prepared as described above, adjusted to OD₆₀₀ = 0.5 (approximately $1\text{--}2 \times 10^8$ CFU/mL), and 20–40 μ L was injected intratumorally per tumor using a 27-gauge needle inserted toward the central tumor mass. One tumor per mouse received ST-Control and the contralateral tumor received ST-pLux-Collagenase, enabling within-animal comparison that controls for systemic immune variation. Tumors were harvested at the study endpoint and processed for histology and mxIF as described below. Animals exhibiting ulceration at the tumor site were humanely euthanized per IACUC protocol and removed from the primary analysis; their tissue was retained for exploratory histological examination.

3.3.6 Masson's trichrome staining and collagen quantification

Harvested tumors were fixed in 10% neutral buffered formalin for 24–48 hours at 4°C, paraffin-embedded, and sectioned at 5 μm . Masson's trichrome staining was performed on deparaffinized sections using a standard protocol (Sigma-Aldrich HT15 kit) per the manufacturer's instructions. Whole-slide images were acquired on a Leica Aperio AT2 whole-slide scanner at 20 $\times$ magnification and exported as .svs files. Computational stain segmentation was performed using a custom Python pipeline applying pixel-level color deconvolution to separate the trichrome blue (collagen), red (cells/muscle), and pale (necrosis/acellular) channels. Adipose tissue regions were manually masked and excluded from analysis. Collagen fraction was calculated as the proportion of non-adipose tissue area classified as collagen-positive (blue channel above threshold). Necrotic fraction was calculated analogously. Collagen density maps were generated by computing collagen pixel fraction within a sliding 100 $\times$ 100 pixel window across the tissue section, producing a spatially resolved heatmap of collagen density. Statistical comparison between treatment groups was not performed given the underpowered pilot cohort; descriptive statistics (mean, IQR) are reported.

3.3.7 Multiplexed immunofluorescence imaging and analysis

Multiplexed immunofluorescence (mxIF) was performed on 5 μm FFPE sections using a sequential tyramide signal amplification (TSA) protocol. The antibody panel included: anti-LPS (Abcam ab35654, 1:200) to identify intratumoral bacteria; anti-CD8 α (Cell Signaling Technology 98941S, 1:200) to identify cytotoxic T lymphocytes; anti-F4/80 (Abcam ab6640, 1:100) to identify macrophages; and DAPI for nuclear counterstain. Sequential rounds of antibody incubation, TSA amplification, and antibody stripping were performed per the Opal mxIF protocol (Akoya Biosciences). Whole-slide mxIF images were acquired on a Vectra Polaris multispectral imaging system and spectrally unmixed using inForm software. Downstream image analysis was performed in Python using a custom pipeline: (i) cellular segmentation

using DAPI-guided nucleus detection; (ii) per-cell marker positivity thresholding; (iii) radial distribution analysis computing mean signal intensity as a function of distance from the tumor geometric centroid, with radial slope calculated by linear regression of intensity vs. distance across 50 μm radial bins; (iv) spatial co-localization scoring calculated as the Pearson correlation coefficient between two marker channels within a sliding 50 μm window across the tissue section, producing the co-localization heatmaps shown in Figure 3.6C. LPS signal intensity per tissue area was used as the colonization proxy metric (Figure 3.6D, colonisation panel).

3.3.8 Experimental design and pilot study limitations

The MC38 in vivo pilot study was designed as a within-animal bilateral comparison, with each mouse serving as its own control by receiving ST-Control in one flank and ST-pLux-Collagenase in the contralateral flank. This within-animal design was selected to maximize statistical efficiency given the inherent variability of subcutaneous tumor models and to control for inter-animal differences in immune response, tumor growth kinetics, and bacterial clearance. The planned cohort size was $n = 8$ mice (16 evaluable tumors), which was calculated to provide 80% power to detect a 40% reduction in collagen fraction with the variance estimated from prior trichrome studies in the MC38 model.

The realized cohort was reduced below this target due to the development of tumor ulceration in a subset of animals, predominantly in the ST-pLux-Collagenase treatment arm. Ulceration at the tumor site is a known complication of high-level intratumoral bacterial colonization and is consistent with the collagenase strain achieving greater ECM disruption than the control strain, a phenotype that, while biologically meaningful and directionally supportive of the mechanistic hypothesis, triggered IACUC humane endpoint criteria that required early euthanasia of affected animals. The disproportionate occurrence of ulceration in the collagenase arm (3 of 5 animals with collagenase tumors vs. 0 of 5 control-only animals, representing a preliminary observation rather than a statistically tested finding) is itself an indirect biological indicator of superior collagenase-mediated ECM disruption in vivo, and is noted as a parameter to

be managed through dose optimization in the planned follow-up cohort. All quantitative analyses of the in vivo dataset are therefore presented as descriptive and exploratory, with directional conclusions drawn conservatively and in the context of the convergent PDO evidence base.

3.3.9 Statistical analysis

Statistical analyses for PDO experiments (CFU, viability, morphologicals, qPCR) were performed as described in Aim 2. One-way ANOVA with Tukey's post-hoc test was used for all three-condition comparisons; significance thresholds are denoted $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ throughout. For the co-inoculation experiment, the same statistical framework was applied independently to therapeutic proxy strain CFU and facilitator strain CFU as separate outcome variables. In vivo data are presented as descriptive statistics only (mean $\pm$ SEM, individual data points shown) and no inferential statistical testing was applied to the MC38 pilot dataset given the underpowered cohort.

3.4 Acknowledgements

Chapter 3, in part, is currently being prepared for submission for publication of the material. The dissertation author was the primary investigator and author of this material.

3.5 Supplementary Data

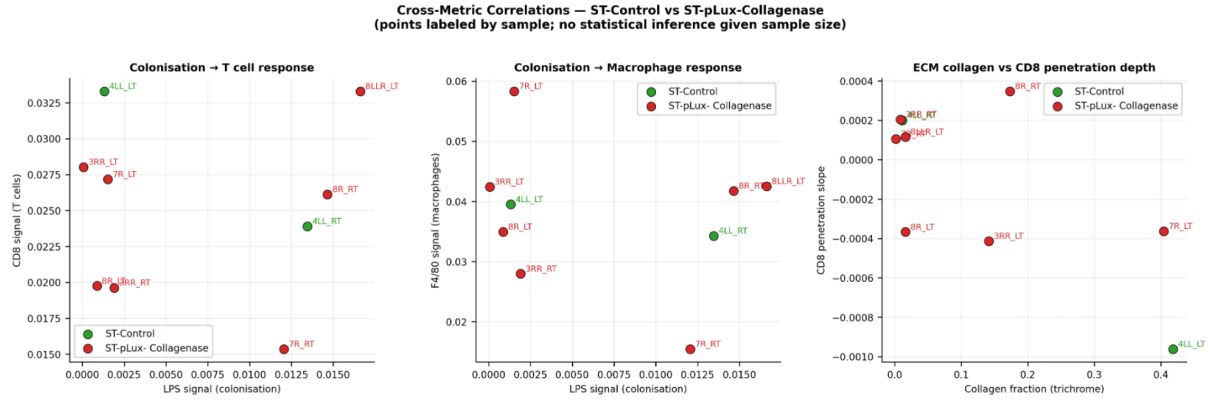


Figure 3.7. Supplementary Figure 1. Cross-metric correlation analysis across ST-Control and ST-pLux-Collagenase tumors. Scatter plots of paired mxIF and trichrome metrics across all ST-Control ($n = 2$, green) and ST-pLux-Collagenase ($n = 6$, red) tumor samples, with individual samples labeled. No statistical inference is applied given sample size. **Left:** LPS colonization signal versus CD8 T cell signal. 4LL_LT (injected ST-Control tumor) occupies the highest CD8 signal position despite near-zero LPS, consistent with a residual immune response at the injection site in the absence of sustained bacterial colonization. 4LL_RT (non-injected contralateral tumor) shows intermediate LPS signal with lower CD8, consistent with early-stage bacterial establishment without a proportionate adaptive immune response. **Centre:** LPS colonization signal versus F4/80 macrophage signal. **Right:** Trichrome collagen fraction versus CD8 radial penetration slope (positive slope indicates immune cells concentrated in the EpCAM-negative tumor core; negative slope indicates peripheral immune exclusion). 4LL_LT is the most extreme outlier in the dataset, combining the highest collagen fraction (0.418) with the most negative CD8 slope (-0.00097), exemplifying the immune-excluded phenotype in a non-collagenase-treated tumor. ST-pLux-Collagenase samples cluster at lower collagen fractions with slopes distributed around zero or positive, consistent with reduced ECM barrier and improved immune access to the tumor interior.

Chapter 4

Discussion and Conclusions

4.1 Overview

In this work, we have engineered a quorum-regulated collagenase circuit into an attenuated *Salmonella typhimurium* chassis and evaluated whether it improves intratumoral bacterial colonization across three experimental systems of increasing biological complexity. In Chapter 1, we establish proof of concept using HCT-116 spheroids, confirming that collagenase expression is metabolically compatible with the bacterial chassis, produces density-dependent ECM disruption, and improves penetration of a GFP-tagged derivative relative to a non-collagenase control at low seeding density. In Chapter 2, we deploy ST and EcN in patient-derived CRC organoids from matched tumor and normal colon tissue and characterize the colonization response across multi-modal readouts including CFU, fluorescence, morphologies, transcription, and secondary organoid formation. In Chapter 3, we evaluate the collagenase strain directly in the PDO system without a fluorescent reporter, demonstrate that it outperforms a vector-matched control across all measured endpoints, and show that it significantly enhances the colonization of a co-administered therapeutic proxy strain. A pilot in vivo experiment in the MC38 syngeneic model provides directional evidence for collagenase-mediated ECM remodeling and improved immune cell penetration that, while underpowered for statistical inference, is consistent with the mechanism established in vitro.

Taken together, the data support a model in which quorum-regulated collagenase expres-

sion lowers the physical barrier to bacterial colonization in a density-dependent, tumor-selective manner, and in doing so creates a more permissive intratumoral environment for a co-administered partner strain. We refer to this as a therapy primer strategy: the collagenase strain is not intended to act as the therapeutic agent, but to condition the tumor ECM ahead of whatever therapeutic intervention follows.

4.2 Quorum sensing as a biosafety mechanism

A core design choice in this work is the use of a quorum-sensing architecture to gate collagenase expression on bacterial population density rather than on exogenous inducers or constitutive promoters. This choice was not primarily motivated by performance (a constitutively expressed collagenase would presumably produce ECM disruption at least as efficiently) but by the recognized toxicity of uncontrolled collagenase activity in non-tumor tissue. Bacterial collagenases are classical virulence determinants because of their ability to degrade connective tissue and facilitate dissemination, and their systemic administration has been associated with off-target tissue damage and immune-mediated adverse effects [42]. Delivering a constitutively expressed collagenase in a tumor-colonizing bacterium would replicate this virulence dynamic intentionally.

The pLux architecture addresses this by coupling enzyme expression to the ecological conditions that define successful intratumoral colonization: high local bacterial density, which only accumulates in the hypoxic and necrotic tumor core that *Salmonella* preferentially colonizes after systemic administration [12, 10]. In normal tissue, bacteria that fail to establish this niche do not reach quorum and therefore do not activate collagenase expression. The matched tumor-versus-normal organoid comparisons across both patient lines and both the ST-GFP and ST-pLux-Collagenase experiments confirm this directly: near-zero CFU in normal colon organoids implies near-zero AHL accumulation, which implies near-zero collagenase expression. The safety margin is not theoretical; it is operationalized through the colonization ecology of the

chassis itself.

For this reason, a constitutive collagenase in vivo control was not included in the MC38 pilot study, as it would be testing a design that is not appropriate for in vivo use, against a design that is. The relevant comparison is between the quorum-regulated construct and an isogenic vector-matched control, which is what was done.

4.3 The PDO co-culture platform: contributions beyond colonization data

The patient-derived organoid co-culture system established in Chapter 2 produces results that are more clinically interpretable than spheroid models for a few practical reasons. The organoids preserve patient-specific mutational context and ECM composition. The matched tumor-normal comparison provides an internal control for tumor selectivity that a monoculture system cannot. And the two patient lines introduce molecular subtype as an experimental variable rather than treating it as a confound to be held constant.

The morphological pipeline developed alongside these experiments is worth discussing as a contribution in its own right. Fluorescence-based bacterial quantification is standard but restrictive: it requires a GFP or similar reporter, imposes metabolic burden, and becomes unusable when the construct occupies all available fluorescent channels or when the strain of interest is untagged for biosafety reasons. The observation that integrated density and raw integrated density achieve $AUC = 0.971$ as classifiers of high ST colonization state from brightfield images alone means that the pipeline can in principle be applied to any bacterial strain, including clinical candidates that cannot carry reporter transgenes. We applied this directly in Chapter 3, where the untagged ST-pLux-Collagenase strain produces a UMAP-separable morphological signature across both patient lines without any fluorescence input. The pipeline's utility is therefore not limited to the specific experimental context in which it was developed.

The cross-patient conservation analyses add something that was not present in the orig-

nal Communications Medicine publication. morphological responses to ST colonization are significantly correlated between the MSS and MSI lines ($r = 0.606$, $p = 0.006$), while transcriptional responses are not ($r = 0.268$, $p = 0.561$). This suggests that the physical consequences of colonization (organoid size reduction, darkening, and structural disruption captured by morphologicals) are more generalizable across patients than the transcriptional programs those same patients mount in response to bacterial presence. If that holds across a larger organoid biobank, it has implications for how bacterial therapy trials should be monitored: morphological readouts of colonization-associated tissue change may be better candidates for cross-patient biomarkers than gene expression signatures, which appear to be heavily shaped by tumor-intrinsic biology.

The divergent conservation of specific transcriptional programs is also informative. *TNF* and *LCN2* are significantly elevated by ST in both patient lines, likely reflecting a stereotyped host response to gram-negative bacterial invasion. EMT markers (*SNAIL*, *VIM*) and metabolic markers (*PDK4*) diverge substantially between lines. In the MSS patient, ST drives strong *SNAIL* induction that is paradoxically suppressed by the collagenase variant; in the MSI patient, *VIM* is downregulated by the collagenase strain in a pattern consistent with loss of matrix-derived mechanical cues that sustain mesenchymal transcriptional programs [36]. These reflect the biological reality that two patients with different tumor architectures, ECM compositions, and mutational histories respond to the same bacterial perturbation in different ways. The platform is sensitive enough to detect that, which is the point.

4.4 MSS vs. MSI differential and patient stratification

The MSI patient line shows attenuated but directionally conserved responses across all three aims. Colonization is lower, morphological effect sizes are smaller, qPCR fold changes are reduced, and secondary organoid viability is not significantly affected. This is consistent with what is known about the MSI TME: higher baseline immune infiltration, a distinct ECM composition arising from mismatch repair deficiency, and therapeutic resistance patterns that

extend across multiple drug classes [32].

This subtype-dependent variation in colonization efficiency has not been well characterized in the bacterial therapy literature, where most studies use a single cell line or mouse strain and generalize accordingly. The present work suggests that molecular subtype should be treated as a stratification variable in bacterial therapy study design, much as it already is in immunotherapy trials. A bacterial therapeutic that performs equivalently to control in an unselected population might achieve significance in a MSS-enriched cohort, and miss entirely in a MSI-enriched one, or vice versa. Two patients is obviously not sufficient to make that claim with confidence.

More practically, the fact that the collagenase primer shows detectable and statistically significant effects in the MSI line despite the overall attenuation is relevant. MSI patients who progress on checkpoint inhibitor therapy have limited subsequent options. If a bacterial ECM primer can meaningfully change the tumor environment even in a therapeutically resistant subtype, it might function as a re-sensitization strategy rather than a standalone treatment. That application is speculative given the current data, but the directional signal is there.

4.5 Enzymatically-active bacteria as a therapy ”primer”

The dominant paradigm in bacterial cancer therapy has been single-strain optimization: find the right chassis, engineer the right payload, demonstrate cytotoxicity [19, 46]. This has produced important proof-of-concept results, but clinical translation has been slow, partly because bacteria at clinically tolerable doses rarely achieve antitumor effects that compete with established therapies [55, 9]. Bacteria are exceptional at reaching the parts of tumors that drugs cannot. They are less exceptional at killing tumors on their own.

The therapy primer strategy asks bacteria to do what they are already demonstrably good at. Rather than engineering a bacterium to deliver a cytotoxic payload that must achieve efficacy as a standalone agent, the collagenase strain is engineered to remodel the physical ECM barrier

that limits both bacterial colonization and immune cell access. The therapeutic consequence is indirect: a more permissive tumor environment that amplifies the performance of whatever comes next (i.e. a co-administered bacterial therapeutic, a checkpoint inhibitor, a chemotherapeutic agent) rather than replacing it.

The co-inoculation experiment in Chapter 3 provides direct experimental support for this concept at the organoid level. ST-pLux-Collagenase significantly enhances ST-GFP colonization in CRC organoids from both patient lines, above and beyond the effect of a vector control primer. The antibiotic-selective CFU quantification confirms that both strains co-exist in the co-inoculation wells and that the enhancement is attributable specifically to the collagenase payload. This is a proof of concept for a division-of-labor consortia architecture: one strain dedicated to ECM remodeling, another dedicated to therapeutic delivery. The two strains do not compete; they cooperate, with the primer strain creating the conditions that enable the therapeutic strain to accumulate.

This consortia design sidesteps some of the metabolic burden problems that arise when a single strain must simultaneously perform multiple functions. A strain that expresses collagenase, a therapeutic payload, and a reporter is under more selective pressure to lose plasmid function than a strain with a single defined role. Splitting the labor across two strains, each optimized for one function, may produce more stable and more tunable performance. The SLC architecture is well-suited to this because the quorum threshold can in principle be tuned independently in each strain, enabling temporal coordination: the primer strain reaching quorum and remodeling ECM before the therapeutic strain reaches quorum and initiating payload delivery. Whether that coordination is achievable in vivo is a question for future work.

4.6 Pilot subcutaneous tumor study implications

The MC38 pilot study needs to be read carefully. All four measured endpoints (colonization by LPS signal, collagen fraction by trichrome, CD8⁺ T cell penetration depth, F4/80⁺

macrophage penetration depth) trend in the direction predicted by the therapy primer hypothesis. The apparent reduction in collagen fraction from 41.8% in ST-Control tumors to 17.3% in ST-pLux-Collagenase tumors is a large difference. The directional improvement in immune cell radial slope is consistent with the physical ECM barrier being reduced. These trends are internally coherent across mechanistically linked endpoints in a way that is unlikely to reflect noise.

What the data do not establish is statistical significance, causal inference about antitumor efficacy, or generalizability beyond the pilot cohort. The ulceration events that reduced effective sample size disproportionately in the collagenase arm introduced a systematic selection bias that cannot be corrected analytically. Animals that ulcerate are removed; the remaining collagenase-treated animals are therefore those in which, for whatever reason, the collagenase strain produced less extreme local ECM disruption. The measured group differences are therefore likely conservative estimates of the true treatment effect, which complicates interpretation but does not reverse the directional conclusions.

The ulceration finding itself is worth discussing. Ulceration at the tumor site following intratumoral bacterial injection is a documented complication of high-level bacterial colonization, and its disproportionate occurrence in the collagenase arm is indirect evidence that the collagenase strain is producing more extensive *in vivo* ECM disruption than the control strain. This is biologically consistent with the *in vitro* data and with the directional trends in the trichrome results. It is also a safety signal that needs to be addressed through dose optimization before a powered follow-up study. The goal is not zero ECM disruption but controlled ECM disruption; enough to improve therapeutic access without destabilizing the tumor to the point of ulceration. That calibration is exactly what the *in vivo* model is suited to inform.

The bilateral colonization asymmetry observed in the ST-Control animal raises a mechanistic point worth noting in the context of model design. Physical disruption of tumor tissue by intratumoral injection constitutes a sterile inflammatory stimulus: needle penetration releases DAMPs that activate resident macrophages and neutrophils, creating an acutely inflamed mi-

croenvironment at the injection site that is relatively hostile to bacterial persistence [20]. Under this model, bacteria that survive the injection-site inflammatory response enter circulation and traffic to the contralateral tumor, which presents an intact hypoxic necrotic niche without the immune priming that impairs colonization at the direct injection site. This sequence is consistent with published observations that attenuated *Salmonella* can disseminate hematogenously and preferentially colonize established tumors through a mechanism dependent on tumor hypoxia and impaired immune surveillance [44, 51].

The more consequential observation is the contrast this creates with the ST-pLux-Collagenase group, in which the same bilateral asymmetry is not apparent. One interpretation is that collagenase-mediated ECM remodeling at secondary colonization sites accelerates immune clearance of trafficked bacteria. In the ST-Control arm, bacteria that reach the contralateral tumor encounter an intact collagen scaffold that physically impedes immune cell infiltration and provides structural cover for bacterial microcolonies [11]. The collagenase strain removes this cover through quorum-regulated enzymatic activity, rendering any site the bacteria traffic to more accessible to macrophage and T cell-mediated clearance. This would reduce net bacterial persistence at both injection and contralateral sites in the collagenase arm relative to the control, consistent with the higher ulceration rate as a consequence of immune activation rather than unchecked colonization. It also suggests that the bilateral model design, in which one tumor is treated and the other serves as an internal control, may require reinterpretation for collagenase-expressing strains: if collagenase modulates immune clearance efficiency at any site bacteria traffic to, the non-injected tumor is not a passive systemic reference but an active participant in the therapeutic response. Future experiments in this model should include LPS imaging at both tumor sites and at serial timepoints to characterize trafficking kinetics independently for each strain.

4.7 Implications and future directions

The most immediate next experiment is a powered MC38 cohort with dose optimization informed by the pilot data. The ulceration-associated attrition suggests that the current bacterial input dose produces colonization above the therapeutic window for some animals. Titrating the dose down while measuring trichrome collagen fraction, immune infiltration, and tumor volume as primary endpoints should be technically straightforward given the infrastructure established here.

Beyond that, several directions emerge naturally from the data.

Combination with checkpoint inhibitors. MSS CRC is largely refractory to anti-PD-1 and anti-CTLA-4 therapy, in part because the TME is immune-excluded rather than immune-inflamed [4]. The directional improvement in CD8⁺ T cell penetration depth in collagenase-treated MC38 tumors suggests that ECM remodeling may partially convert the immune-excluded phenotype toward immune-inflamed. If that conversion is sufficient to sensitize MSS tumors to checkpoint blockade, the combination of a collagenase primer with anti-PD-1 could be tested in the MC38 model, where baseline checkpoint inhibitor response is known. This experiment would directly test whether the primer concept translates to improved efficacy of an existing approved therapy.

Combination with conventional chemotherapy. The same collagen matrix that limits bacterial penetration limits small-molecule drug diffusion into the tumor core [52]. Enzymatic depletion of tumor collagen has been shown to improve chemotherapeutic penetration and response in preclinical models of pancreatic cancer [15], and the principle should extend to CRC given the shared desmoplastic biology. Measuring intratumoral oxaliplatin or irinotecan concentration as a function of trichrome collagen fraction in the MC38 model would be a tractable way to ask whether the collagenase-mediated collagen reduction observed in the pilot translates to improved drug delivery.

Extension to other desmoplastic tumor types. Pancreatic ductal adenocarcinoma

is the canonical desmoplastic malignancy, and the degree of ECM barrier problem in PDAC substantially exceeds what is seen in CRC. *Salmonella* has been shown to colonize orthotopic pancreatic tumors in mouse models [6], and the collagenase circuit is in principle portable to that context. Whether the pLux quorum threshold is achievable in the PDAC TME, given its distinct hypoxia and acidosis profile, would need to be established using a PDAC-adapted version of the TME stressor screening performed in Chapter 2. The PDO platform is directly adaptable to pancreatic organoids with appropriate media formulation.

Consortia architecture development. The co-inoculation experiment establishes proof of concept for a two-strain division-of-labor system. Moving from proof of concept to a functional therapeutic strategy requires characterizing the stability of both strains in co-culture over time, establishing that neither strain out-competes the other for intratumoral space, and determining whether quorum threshold timing can be offset between strains to achieve sequential primer-then-payload delivery. These questions are addressable in the BSCC system before committing to in vivo experiments.

Expansion of the organoid biobank. Two patients is a case study. Whether the MSS/MSI colonization differential generalizes, whether the conserved inflammatory core and patient-specific EMT pattern holds across a broader patient population, and whether the morphological pipeline performs consistently across organoids from patients with different prior treatment histories, these questions require a larger biobank. The infrastructure for this exists; it is a matter of continued tissue access and the processing time to establish and characterize additional lines.

CAR-T and adoptive cell therapy combinations. The physical trafficking barrier that limits CAR-T efficacy in solid tumors is mechanistically similar to the one that limits bacterial penetration [48]. A bacterial ECM primer administered before CAR-T infusion could in principle improve T cell access to the tumor without requiring modification of the cell product. This is further from the current experimental infrastructure than the other directions listed here, but it follows from the same logic and represents a high-value clinical application given the unmet

need in solid tumor CAR-T therapy.

4.8 Limitations

The absence of an immune system in the PDO experiments is the most significant biological limitation of the in vitro data. The qPCR results show that the organoid epithelium mounts an inflammatory transcriptional response to bacterial presence, but immune-mediated clearance, immune-bacterial crosstalk, and the contribution of immune cells to ECM remodeling are all absent from the organoid co-culture. The MC38 model partially addresses this, but the underpowered cohort limits what can be concluded from it. Immune-competent in vivo studies with sufficient n remain the necessary next step before mechanistic claims about immune infiltration can be made with confidence.

The two-patient organoid dataset supports the cross-patient conservation analysis as a proof of concept, but the MSS and MSI categories each contain only one patient. Individual variation within each molecular subtype could account for some of the observed differences between lines. Expansion to five or more patients per subtype, with appropriate clinical annotation, is needed before the stratification pattern can be treated as a generalizable finding rather than an observation.

Quorum threshold attainment in vivo has not been directly measured. The in vivo evidence for collagenase expression is indirect (reduced collagen fraction by trichrome staining) rather than direct quantification of collagenase protein or activity in tumor tissue. It is possible that some of the collagen reduction reflects tumor necrosis associated with bacterial colonization rather than enzymatic degradation specifically. Distinguishing these mechanisms would require collagen fiber architecture analysis and potentially activity-based collagenase probes in the tissue, both of which are planned for the follow-up study.

Finally, the regulatory pathway for intratumorally administered engineered bacteria carrying synthetic gene circuits is still being defined. The containment strategy employed here,

gentamicin-mediated confinement to the tumor core, supported by the quorum threshold for payload activation, provides a rational biosafety framework, but translation to a clinical setting will require engagement with regulatory agencies well before first-in-human studies.

4.9 Conclusions

This work began from the observation that the ECM barrier is as much a limiting factor for bacterial cancer therapeutics as it is for drugs and immune cells, and asked whether bacteria could be engineered to remove that barrier from within. The data across three experimental systems suggest they can, at least in vitro and at least directionally in vivo, when collagenase expression is gated on the population density conditions that define intratumoral colonization.

The broader argument is that bacteria need not be the therapeutic agent to be therapeutically useful. A bacterium that conditions the tumor ECM for improved access by other interventions (i.e. existing chemotherapy, existing checkpoint inhibitors, existing cellular therapies) has clinical value without needing to cure cancer on its own. This is, in some ways, a more modest claim than what the field has historically pursued. It is also, for that reason, more tractable. The treatments that already exist work when they can reach the tumor. The question is how to help them get there.

The collagenase primer is one answer to that question. Whether it is the right one, and in which patients, and in combination with which therapies, are questions that the data here motivate but do not resolve. Moving from motivating those questions to answering them is the work that remains.

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