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Engineered bacterial therapeutics for detecting and treating CRC

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Abstract

Despite an overall decrease in occurrence, colorectal cancer (CRC) remains the third most common cause of cancer deaths in the USA. Detection of CRC is difficult in high-risk groups, including those with genetic predispositions, with disease traits, or from certain demographics. There is emerging interest in using engineered bacteria to identify early CRC development, monitor changes in the adenoma and CRC microenvironment, and prevent cancer progression. Novel genetic circuits for cancer therapeutics or functions to enhance existing treatment modalities have been tested and verified *in vitro* and *in vivo*. Inclusion of biocontainment measures would prepare strains to meet therapeutic standards. Thus, engineered bacteria present an opportunity for detection and treatment of CRC lesions in a highly sensitive and specific manner.

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The history of bacterial treatment of cancer

Interest in engineered **live bacterial therapeutics (LBTs)** (see Glossary) has been growing due to their advantages over traditional therapeutics, especially in addressing difficult-to-treat diseases [1]. Unlike traditional probiotics, which aim to make compositional changes to the **microbiome,** engineered LBTs can be modified to produce specific compounds of interest, making a functional change in the microbiome [2]. Bacteria have a natural propensity to synthesize bioactive molecules and can do so *in situ* [1]. Local production and delivery of therapeutics can increase efficacy of treatment, notably by decreasing systemic levels which subsequently reduces side effects [1]. Additionally, engineered LBTs can deliver compounds that are otherwise too unstable for oral delivery [3,4], because bacteria are well adapted to sense and respond to the local environment [2], and the detection of specific environmental conditions can be used to trigger the expression of therapeutic agents. Engraftment of a sense-and-response engineered LBT at a location of interest could allow for long-term therapeutic administration that occurs only when necessary, tailoring dosage and frequency to the severity of disease pathology [2,4–6]. Furthermore, this sense-and-response function could be used as a noninvasive approach for detecting and monitoring disease [2,4]. Overall, the use of engineered LBTs presents a promising option that is currently being tested in preclinical mouse models for the targeted diagnosis and treatment of long-term diseases such as cancer.

Bacteria have long been used in cancer treatment, from 19th century ‘anticancer vaccines’ consisting of heat-killed bacteria to today’s Bacillus Calmette–Guerin vaccine for the treatment of non-muscle invasive bladder cancer [7]. Previous methods relied on probiotic bacteria to naturally increase immune response in the **tumor microenvironment**, to deplete the tumor of needed nutrients, or to naturally generate products that negatively affect tumor cells [7,8]. Now, LBTs can be engineered to produce targeted anticancer payloads such as cytotoxic proteins, immunomodulators, or small-molecule inhibitors [8–10]. Improved bacterial **chassis** can be identified from bacterial strains that are increasingly associated with different tumor types [8–13], and they can be engineered to further increase their ability to home in on the tumor microenvironment [9]. Indicators of the tumor microenvironment – such as decreased pH and increased inflammatory markers – can trigger therapeutic production, increasing delivery selectivity and decreasing adverse effects on healthy tissues [8,9]. Biosensors with detectable outputs (e.g., fluorescent, colorimetric, or luminescent) can be used for cancer detection and to monitor the state of the tumor, success of localization, efficacy of treatment, or cancer metastasis [8]. Thus, the combination of tumor targeting, environment sensing, therapeutic production, and detectable outputs makes LBTs a strong tool for cancer treatment and diagnostics.

C olorectal cancer and prognosis

In the case of CRC, adenoma-to-carcinoma progression in the colon (Figure 1A) may take 5–10 years, making early detection and treatment of CRC by engineered LBTs attractive [10,14]. Early CRC diagnosis (stages 0–2) has an 80% 5-year survival rate, while late-stage diagnosis (stage 4) has only a 10% survival rate [10]. Unfortunately, the risk of

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CRC is still difficult to predict, as <20% of CRC is hereditary, and traditional screening requires colonoscopies which can be costly and invasive [10]. Using the knowledge of the CRC-associated microbiome, LBTs can be developed as simple and noninvasive screening methods for early detection of CRC, as well as a means for targeted CRC treatment [15,16]. In this opinion article we (i) explore the CRC tumor microbiome and microenvironment markers, (ii) discuss potential bacterial chassis for therapeutic delivery, and (iii) address **biocontainment** measures that can be taken to address potential safety concerns.

The tumor microbiome

Bacteria have long been associated with human tumors, but it was previously difficult to characterize individual tumor microbiomes due to low biomass [11]. Recent studies have demonstrated significant variations in the microbiomes of different tumor types, though the exact role microbes play in tumor development and progression remains unclear [11,12]. Bacteria are present in numerous tumor and healthy tissue samples, and immune cells and cancer cells often harbor intratumor bacteria which can be detected by culturing from tumor biopsies to confirm the presence of live bacteria [11]. Furthermore, plasma-derived, cell-free microbial nucleic acids can be used to distinguish between healthy-versus-cancer and cancer-versus-cancer signatures [12,13,17].

Tumor-specific microbiome compositions likely correlate with individual tumor microenvironments. The hypoxic, necrotic, and immunosuppressive regions of tumors are favorable for bacterial growth, and irregular organization of blood vessels in tumors allows for bacterial infiltration (Figure 1B) [7,9]. The tumor microenvironment also provides the specific set of conditions under which therapeutic expression or diagnostic output could be induced. In CRC, inflammation is a major contributor to the tumor microenvironment [14]; COX-2 is overexpressed in the majority of patients with CRC, leading to increased levels of prostaglandins such as PGE2 [14]. Markers of inflammation, such as nitric oxide [9] and hypoxia, could be used to regulate therapeutic expression and delivery. Other markers, such as lowered luminal pH, which has been associated with higher tumor yields in chemical induction of colon carcinogenesis in rodents, can also be used [18]. The tumor microenvironment also indicates potential therapeutic strategies, such as counteracting the conditions correlated with CRC growth and development. For example, an engineered LBT that detects immunosuppression biomarkers and delivers immunostimulatory molecules in response could promote immune-mediated tumor destruction.

The CRC microbiome has been of particular interest due to the potential role of the gut microbiome in driving CRC development (Figure 1C). Patients with CRC often have changes in the composition and function of the gut microbiome [18,19]. Bacteria can play a role in tumor development through their metabolites, through adhesion, invasion, and translocation across cell layers, and by modulating host defense and immune responses [20]. There has been much speculation regarding the ‘driver–passenger’ model of CRC development – one bacterium ‘drives’ carcinogenesis, disappears, and another bacterial ‘passenger’ is enriched to continue the carcinogenic process [19] – but the exact interactions between bacteria and tumorigenesis still need further clarification. Certain bacteria, such as *Fusobacterium nucleatum* or genotoxic polyketide synthase positive (*pks+*) strains of

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Escherichia coli, are predominant microbes associated with CRC and able to localize to the tumor microenvironment [20,21]. While many of these strains contain virulence genes [21–23], their identification provides information on other functions that could be used to target nonpathogenic bacterial strains to the tumor microenvironment. Alternatively, the pathogenic microbes could be isolated and attenuated to eliminate virulence while maintaining their targeting ability. They would then be viable chassis for CRC-specific localization and delivery of anticancer payloads. Overall, understanding the specific tumor microenvironment and microbiome provides insight into chassis choice, biomarkers for detection, and potential therapeutic treatments.

Choosing a bacterial chassis

When designing an engineered LBT, one of the most important choices is what chassis – or bacterial strain that serves as the host or platform for genetic modifications and engineering – is best to use [24]. First, there must be genetic tools available to alter the bacteria to perform the function of interest. Next, the bacteria must be capable of producing and delivering the therapeutic agent or contain a functional sensor for the disease state. In addition, a good chassis must be able to survive in the target environment long enough to sense the conditions and/or respond accordingly. Finally, an ideal chassis should exhibit **genetic stability**, minimizing the risk of both horizontal transfer of target genes, which can pose biocontainment concerns, and the inadvertent deletion of transformed genes. However, genetic stability in a bacterium, while advantageous, can also pose challenges in terms of its manipulability. Depending on the intended function, one must consider whether the bacteria can colonize and persist at the targeted site. The long-term engraftment of an LBT could allow the monitoring or treatment of long-term or chronic diseases [1,4].

Most LBTs use probiotic bacterial strains that are 'Generally Regarded as Safe' by the US Food and Drug Administration, such as *Lactococcus lactis* strains or *E. coli* K-12 [25] (www.fda.gov/food/generally-recognized-safe-gras/microorganisms-microbial-derived-ingredients-used-food-partial-list). Other well-studied bacteria, such as *E. coli* Nissle 1917 and *Bifidobacterium* spp., are also commonly used. However, the same reasons that probiotics are considered safe often contribute to their inability to colonize humans. While these engineered probiotics can deliver therapeutics, their potential for excretion and inability to engraft results in the requirement of high-dose and frequent administration. This may be sufficient for the sensing and treatment of acute diseases, but for treating or preventing diseases such as CRC, a stably colonizing bacterium may be a better choice [26]. A bacterium isolated from the target environment may be the ideal chassis. Native *E. coli* have consistent and durable engraftment in an intact gut microbiome, while probiotic strains can be quickly cleared and colonization varies among individuals [27,28]. Additionally, though editing efficiency of native *E. coli* is lower than that of *E. coli* Nissle 1917, resistance to editing could be indicative of genetic stability, which is favorable in long-term LBT development [29].

In the context of CRC, a bacterial chassis must be well suited to the luminal environment of the intestines but also specific to the microenvironment of CRC tumors. Serendipitously, *E. coli*, the bacterium with the most developed synthetic biology tools, is commonly

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associated with CRC in both humans and mice [20]. With numerous verified gene induction methods, genetic engineering techniques, and the ability to produce therapeutic molecules, designing an engineered LBT using *E. coli* is well supported in the literature [27]. While *pks*+ *E. coli* is implicated in CRC tumorigenesis, nonpathogenic *E. coli* is regularly found in the normal gut microbiome [30–32]. Furthermore, *E. coli* Nissle 1917 is a probiotic that has been widely used in the development of LBTs [1,4,33] and can home in to cancerous tissue, including CRC tumors [16,34]. However, *E. coli* Nissle 1917 is also a *pks*+ strain, and could thus exacerbate CRC development unless appropriately attenuated. Recent publications indicate that attenuation of the *pks* island does not prevent localization of *E. coli* Nissle 1917 to CRC tumors, with tumor-enriched colonization monitored up to 1 month post-administration [16]. But, due to its variable colonization capacity in healthy humans, typically decolonizing after cessation of daily administration [4,35], this strain is likely better suited to transient detection of the disease state or treatment efficacy, rather than prolonged monitoring, treatment, or repression of CRC development over the timespan of months or years.

Alternatively, native bacteria possess strong colonization capabilities and can be engineered as LBTs [6,26,27]. Engineered strains of commensal *E. coli* durably engraft in mice and maintain functionality; they have been used diagnostically for intestinal inflammation detection over a 6-month period [6] and for therapeutic treatment of hyperglycemia 3–4 months after administration of the LBT [26]. Isolating *E. coli* directly from a CRC tumor would ensure that the chassis has developed the ability to survive in and to home in on tumors. Strains of *pks*+ *E. coli* are associated with CRC development, likely due to their production of colibactin, a DNA alkylating agent that creates double-strand breaks and cross-links DNA [31]. When this occurs in the presence of host inflammation, it accelerates colon tumor growth [31]. However, if the critical peptidase enzyme ClbP is knocked out of the *pks* virulence island, the genotoxin cannot be made [31] and tumorigenesis is no longer evident [27]. Thus, attenuating the virulence of a native strain of *pks*+ *E. coli* that is enriched in tumors could produce a chassis that is highly specific to CRC tissue. Similarly, enterotoxigenic *Bacteroides fragilis* is associated with CRC and works with *pks*+ *E. coli* to drive CRC tumorigenesis. As *Bacteroides* spp. are among the most stable constituents of the human gut microbiome, and recent work has expanded the genetic toolset for engineering them [36], an attenuated *B. fragilis* from the tumor microbiome or other commensal gut *Bacteroides* could serve as an ideal chassis for the long-term treatment or monitoring of CRC. However, since *B. fragilis* does not engraft in the gut of most mice with complex microbiomes, it may be difficult to perform preclinical studies testing the effectiveness of this particular chassis [37]. Other commensal bacteria that are commonly found in the gut microbiome could also be explored as stably colonizing chassis, such as bifidobacteria [38], lactococci [25], or lactobacilli [25].

Payload delivery

The efficient delivery of solid tumor therapies poses a unique set of challenges, primarily stemming from the need to transport therapeutic molecules without compromising their functionality. Previous studies have used chemical inducers, optogenetics, thermal regulation, and sensing of tumor-specific conditions such as oxygen and glucose gradients

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for the controlled release of therapeutic payloads [39]. Researchers can also harness native bacterial quorum sensing systems to trigger delivery of solid tumor therapies. Quorum sensing is a cell-to-cell communication process using small molecules or oligopeptides for the regulation of bacterial community behaviors such as biofilm formation or virulence factor secretion [40,41]. In Gram-negative bacteria, acyl homoserine lactone (AHL) is used as the primary quorum sensing molecule. AHL proteins can bind to R-proteins, which are DNA-binding transcription factors, to directly regulate gene transcription. For example, in the *Vibrio fischeri* lux quorum sensing system, its AHL 3-oxo-C6-HSL binds with its R-protein LuxR to express a protein of interest only when the bacterial density reaches a certain threshold. Quorum sensing has been used to develop synchronized oscillators [42–44] and a bacterial synchronized lysis circuit (SLC), which triggers synchronous lysis at a threshold population density (Figure 2). Upon synchronized lysis, a small number of surviving cells reseed the population, allowing the SLC cycle to repeat [45]. Given that the SLC system enables external delivery of any protein without requiring an export system, it has proved to be an attractive platform for the delivery of cancer therapies [46,47]. However, the lysis dynamic is a stressor, causing bacteria harboring the SLC to tend to mutate and lose functionality. The stability of the circuit can be increased through a rock–paper–scissors system. In this three-strain system, each strain contains a specific *E. coli*-targeting toxin–antitoxin module along with the SLC such that each strain can kill the previously administered strain but will itself be killed by the subsequent strain in the cycle. For example, a functional SLC 2 strain can completely replace a nonfunctional SLC 1 strain because the SLC 2 strain produces a colicin to which the SLC 1 strain is susceptible. When the SLC 2 strain mutates, it can be completely replaced by the SLC 3 strain, which can then be replaced by SLC 1. This not only lengthens the period of therapeutic delivery, but also allows for the delivery of multiple therapeutics over time [48]. Two different SLCs have been developed using lux and rpa quorum sensing systems. Unlike the lux system, the rpa system uses p-coumaroyl-HSL as its AHL molecule, which binds to RpaR for transcription activation. Thus, the two systems are orthogonal, allowing for the long-term co-culture of metabolically competitive strains [49]. Additionally, scientists have developed an rpa-based inducible SLC (iSLC) that is responsive to both population density and the inducer molecule p-coumaric acid (pCA). The iSLC can transition between inactivated population growth, cyclical cargo release, and global population death [50].

In vivo applications of quorum sensing systems in cancerous mouse models show that threshold population density and subsequent protein production occurs only upon the colonization of cancerous tissue and remains uninduced in the liver [51]. Attenuated *E. coli* and *Salmonella* strains have been engineered to release therapies at a threshold population density using the SLC system, allowing for periodic delivery of a chemokine, hemolysin, pro-apoptotic protein, or all three, into the tumor microenvironment. Researchers have tested many applications of SLCs in relevant cancer models and have demonstrated effective therapeutic delivery, resulting in subsequent tumor reduction. Nanobody targeting of antiphagocytic receptor CD47 [52] or immune checkpoint inhibitors PD-L1 and CTLA-4 [53] combined with the immunostimulatory effect of bacterial lysis products led to rapid and durable regression of tumors, and release of synthetic antigens targeted chimeric antigen

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receptor T cells (CAR-T cells) more effectively to tumors [54]; production of chemokine CXCL16 recruited activated T cells and expanded effector T cells *in vivo* [55].

Overall, the synchronized lysis system provides an appealing clinical approach to the continuous and localized release of therapeutics, preventing systemic toxicity despite an increase in intratumoral concentrations. The lysis circuit design can be used to produce a large variety of cytotoxic payloads, combined with other cell-based therapies, and applied to patient-specific and tumor-specific commensal strains for a personalized treatment regimen for CRC. Since pCA is a molecule that is present in most fruits and vegetables and has been proved safe for human cells, the iSLC also provides an attractive framework for further pursuit *in vivo*. As most Gram-negative strains employ AHL–R-protein quorum sensing systems, the SLC can be adapted to other strains, including *Bacteroides* and *Pseudomonas* spp. Meanwhile, quorum sensing systems in Gram-positive bacteria are less characterized and often involve small oligopeptides that require post-translational processing [56]. Thus, employing an SLC circuit in bacteria such as *Bifidobacterium, Lactobacillus*, or *Lactococcus* spp. may be more difficult.

Inter-individual biocontainment

Genetically modified organisms (GMOs), such as engineered LBTs, require biocontainment safeholds to prevent their spread into unintended environments or recipients. Though there is no direct evidence that released GMOs negatively impact the environment and ecosystems, there is significant concern about their interactions [57]. Criteria set forth by the National Institutes of Health (NIH) calls for an ideal escape rate below 1 in 10^8 cells [58]. Current bacterial therapies, including most live bacterial vaccines and oncolytic therapies, utilize nonreplicating bacteria and inoculate patients with 10^6–10^7 colony forming units [59], minimizing concern of escapee cells. However, this is not applicable to replicating bacteria intended for long-term CRC treatment and monitoring, as they would continue to grow and divide, potentially reaching or surpassing 10^8 colony forming units and increasing the risk of escapee cells. Thus, other measures of bio-containment are especially important for replicating live bacterial therapies.

Common methods of biocontainment largely fall into one of two categories: auxotrophies and kill switches [57–64]. Auxotrophy – the reliance of the bacterial chassis on exogenously provided metabolites due to deletion of an essential gene – is an easy method to institute and can work effectively in the short term. However, a major drawback of making a bacterium auxotrophic for an exogenously provided metabolite is that it can easily be overcome by microbial cross-feeding or the presence of the metabolite in the environment [59,63]. Alternative approaches focus on deleting genes whose functions cannot be complemented by cross-feeding [59] or by creating auxotrophic bacteria that rely on synthetic small molecules [58]. Unfortunately, auxotrophies are largely prohibitive of LBT survival *in vivo* due to limitations regarding what exogenous molecules can be safely administered to patients and will achieve the required concentration to promote cell survival. Furthermore, an auxotrophy can still be overcome through horizontal gene transfer of a functional gene copy from another microbe.

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Alternatively, kill switches – genetic circuits that result in cell death under certain conditions – are more versatile forms of biocontainment [63]. Most kill switches regulate the expression of essential bacterial genes (endogenous genes whose lack of function cannot be overcome by providing an exogenous metabolite), allowing cell survival only in a 'permissive environment' based on a detectable input [63]. An example of an input could be environmental temperature: the expression of an essential gene is repressed at lower temperatures (such as typical 'room temperature') so survival is only possible at the higher temperature within the mammalian body. A kill switch relying on permissive environmental inputs can be applied to replicating LBTs, but the escape rate is often high due to the metabolic burden maintaining and expressing the genetic circuits. Indeed, it has been observed that many kill switches evolve to lose functionality within days [63]. Creating a multilayered genetic circuit decreases the frequency of escape by requiring more mutations to overcome the kill switch, but this is time-consuming for the researcher to construct and is still burdensome to the LBT [59]. Alternatively, kill switches can use inducible expression of lethal genes as a more targeted method of cell killing [59,63]. Combining multiple methods of triggering cell death makes the kill switch useful in both preventing the spread of an LBT and clearing an LBT from a patient's body.

Intra-individual biocontainment

When considering a replicating LBT, the issue of 'intra-individual biocontainment' arises: that is, how can an engrafting LBT be cleared from a patient so treatment can be discontinued at any time? With respect to this issue, kill switches that directly induce cell death (triggering expression of a lethal product as opposed to controlling expression of a gene essential to bacterial survival) are currently the best form of biocontainment for engrafting LBTs. Instead of monitoring for a permissive environment, as described earlier, lethal gene expression can occur within the permissive environment when triggered by exogenous inputs. Lethal kill switches often comprise a toxin–antitoxin system. To alleviate the fitness burden of potential low-level leaky toxin expression, a corresponding molecular 'sponge' is constitutively expressed that inactivates the toxin [55,57,61]. Upon induction with the exogenous molecule, toxin expression exceeds the amount of antitoxin being expressed, causing cell death. However, further work on biocontainment methodology is required, as toxin–antitoxin-based kill switches are still subject to high mutation rates, and *in vivo* use of exogenous molecules for induction is limited [65]. To most effectively deploy colonizing LBTs for CRC monitoring and treatment, a stable kill switch with low fitness burden that can be induced *in vivo* has yet to be developed and thoroughly tested.

Concluding remarks

In conclusion, LBTs provide a unique platform for the detection and treatment of CRC, though there remains much foundation to be established (see Outstanding questions). Particularly, native bacteria offer the potential for long-term, noninvasive CRC detection and treatment, being well adapted to the harsh luminal environment. Tumor microenvironment conditions can be used as inputs to induce the expression of detectable markers (e.g., fluorescence, luminescence) or therapeutic molecules, though detection of multiple environmental conditions may be required to prevent off-target response to other conditions

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(such as ischemia or viral induction of inflammation). Finally, any successful LBTs can be further engineered with a multitude of biocontainment systems to increase safety profiles for translation into clinical trials. The landscape of CRC detection and treatment is rich, ideal for improved patient experiences and outcomes.

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Declaration of interests

A.Z. has a patent for PCT/US18/27998 pending and licensed to Endure Biotherapeutics. A.Z. holds equity in and is the acting Chief Medical Officer of Endure Biotherapeutics. J.H. is a co-founder of GenCirq Inc, which focuses on cancer therapeutics. He is on the Board of Directors and has equity in GenCirq. His spouse is employed part time for the book-keeping and to support employees with Human Resources. The remaining authors have no interests to declare.

Glossary

Biocontainment

prevention of the spread of the live bacterial therapeutic or its genetic material to the environment or other patients

Chassis

in the context of genetic engineering and synthetic biology, the organism (such as a bacterium) that physically houses genetic components and provides the resources needed for them to function (e.g., materials for transcription and translation of the product)

Genetic stability

the frequency of DNA sequence alteration due to spontaneous mutagenesis or DNA transfer into and out of the cell

Live bacterial therapeutics (LBTs)

a therapeutic product that contains living bacteria to prevent, treat, or cure disease, but that is not a vaccine

Microbiome

the community of microorganisms living in a specific environment, such as the gut

Tumor microenvironment

the ecosystem surrounding a tumor, including local noncancerous cells, extracellular signals, and physiological conditions (such as pH, oxygen concentration, and nutrient availability) that affect tumor growth and development

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Highlights

Different cancer types are associated with unique, detectable microbiomes.

Engineered bacteria can be used for cancer detection.

Native bacteria can be engineered and reintroduced into an intact gut microbiome, producing long-term physiological changes in the host.

Probiotics can be engineered with genetic circuits to deliver antitumor payloads and to stimulate host antitumor immunity, and can complement existing cancer therapies such as chimeric antigen receptor T cells (CAR-T cells) to increase treatment efficiency.

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Outstanding questions

What is the relationship between certain bacterial species and the development and/or progression of CRC?

How does the genetic stability of commensal bacteria compare with that of their probiotic strain counterparts?

Does attenuating virulence of cancer-associated bacteria decrease their ability to home in to the tumor microenvironment?

How can probiotic bacteria be engineered to increase tumor homing in strains that are ‘generally regarded as safe’?

What biocontainment measures are effective and stable when using live bacterial therapeutics to treat humans?

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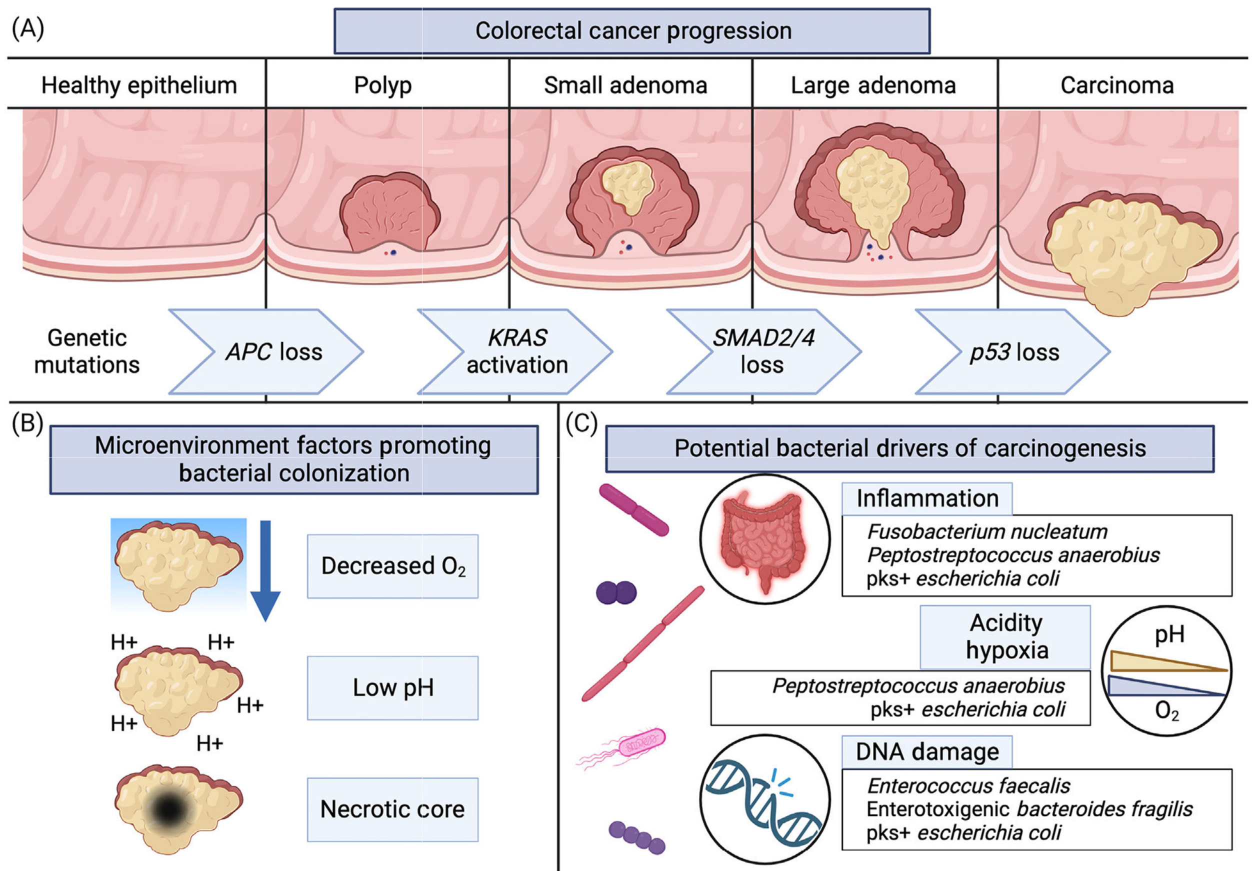

Figure 1. Interactions between the tumor and its microbiota.

(A) Colorectal cancer progression from healthy epithelium to carcinoma. (B) Decreased oxygen, lower pH, and the necrotic core of a tumor are all microenvironmental factors thought to promote colonization of the tumor by certain bacteria. (C) Potential bacterial drivers of carcinogenesis are thought to cause inflammation, decreases in pH and oxygen concentrations, and DNA damage. Abbreviation: *pks*+, polyketide synthase positive.

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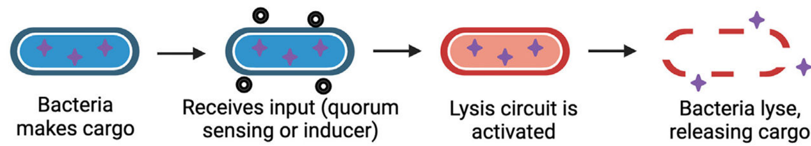

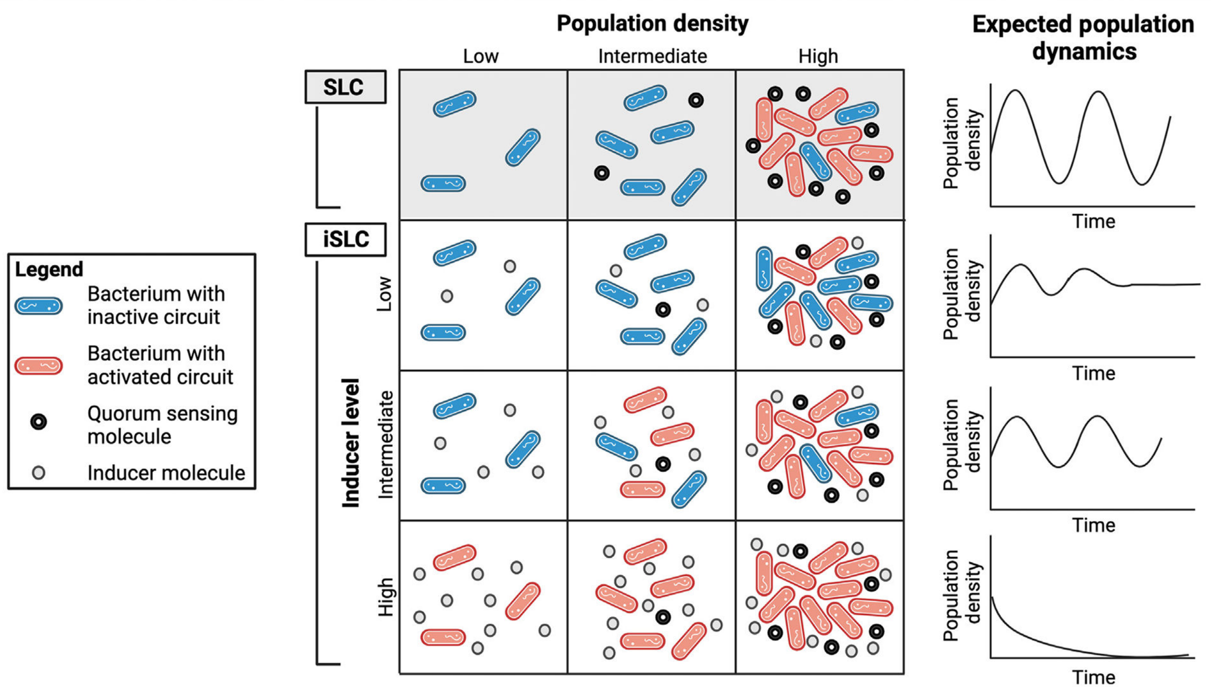

Figure 2. The synchronized lysis circuit (SLC) [39] relies on a sufficiently high density of bacteria leading to the accumulation of quorum sensing molecules, causing synchronized cell lysis.

The inducible SLC (iSLC) includes an additional exogenous inducer molecule that can be applied, modulating the population density requirements for subsequent synchronized lysis.