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Motility is Critical for Effective Distribution and Accumulation of Bacteria in Tumor Tissue

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Abstract

Motile bacteria can overcome the penetration limitations of cancer chemotherapeutics because they can actively migrate into solid tumors. Although several genera of bacteria have been shown to accumulate preferentially in tumors, the spatiotemporal dynamics of bacterial tumor colonization and their dependence on bacterial motility is not clear. For effective tumor regression, bacteria must penetrate and distribute uniformly throughout tumors. To measure these dynamics, we used an in vitro model of continuously perfused tumor tissue to mimic the delivery and systemic clearance of *Salmonella typhimurium* strains SL1344 and VNP20009, and *Escherichia coli* strains K12 and DH5 α . Tissues were treated for 1 hour with 10^5 or 10^7 CFU/ml suspensions of each strain and the location and extent of bacterial accumulation was observed for 30 hours. *Salmonella* had 14.5 times greater average swimming speeds than *E.coli* and colonized tissues at 100 times lower doses than *E.coli*. Bacterial motility strongly correlated ($R^2 = 99.3\%$) with the extent of tissue accumulation. When inoculated at 10^5 CFU/ml, motile *Salmonella* formed colonies denser than 10^{10} CFU/(g-tissue) and less motile *E.coli* showed no detectable colonization. Based on spatio-temporal profiles and a mathematical model of motility and growth, bacterial dispersion was found to be necessary for deep penetration into tissue. Bacterial colonization caused apoptosis in tumors and apoptosis levels correlated ($R^2 = 98.6\%$) with colonization density. These results show that motility is critical for effective distribution of bacteria in tumors and is essential for designing cancer therapies that can overcome the barrier of limited tumor penetration.

Keywords

Bacterial cancer therapy; microfluidic device; spatiotemporal; dispersion; apoptosis

Introduction

Bacterial cancer therapies have the ability to overcome some of the fundamental limitations of cancer chemotherapies, but the role of motility in bacterial penetration into tumors is not clearly understood. Chemotherapeutics are limited by passive diffusion into solid tumors and fail to reach all parts of tumors in sufficient concentrations^{1–4}. Bacteria can penetrate deeper

into tumors and accumulate at higher concentrations because of their ability to actively migrate⁵⁻⁶ and replicate in favorable environments⁷. In animal models, several genera of bacteria have been shown to preferentially accumulate in tumors compared to other organs⁸⁻¹⁴ with greater than 1000-fold selectivity. This accumulation led to tumor shrinkage and enhanced survival¹⁵. Bacteria have also been engineered to express anticancer agents for targeted delivery to tumors^{8, 16-17}. *Salmonella typhimurium*, strains SL1344¹⁸ and VNP20009^{8, 14, 19}, and *Escherichia coli*, strains K12²⁰ and DH5 α ²¹, are facultative anaerobic enteric bacteria with well-studied and easily modifiable genetics that have previously been evaluated as anti-cancer therapies.

To clarify the role of motility there is significant need to quantify the spatiotemporal dynamics of the accumulation of highly and minimally motile bacterial strains in tumor tissue. For bacteria to be effective cancer therapeutics, it is necessary that they penetrate deep into tissue and colonize homogeneously throughout tumors²². It has been shown that bacteria that can better disperse throughout tumors have an increased ability to regress tumors²³. However, bacterial accumulation in tumors is known to be spatially heterogeneous and often restricted to necrotic regions^{14, 23}. In addition, *Salmonella* chemotaxis towards chemicals produced in the necrotic regions of tumors⁵⁻⁶. An attenuated non-pathogenic strain of *Salmonella* (VNP20009) was tested in three Phase-1 clinical trials and found to only colonize tumors in a fraction of patients and not slow tumor growth²⁴⁻²⁶. In one clinical trial, a tumor excised from a patient showed 11,000 CFU/g-tumor bacterial accumulation, but a fine needle aspirate drawn from the same tumor showed no accumulation²⁵, suggesting that the bacterial accumulation within the tumor was spatially heterogeneous. Because there was no reduction in tumor size reported in these studies, the extent of bacterial accumulation was insufficient. To overcome these limitations, it is necessary to understand the mechanisms that control bacterial spreading and accumulation in tumors.

Two mechanisms have been proposed as significant causes of bacterial tumor colonization and subsequent spreading: motility and preferential growth within the tumor microenvironment^{13, 27-28}. Motility is widely accepted as a virulence factor for pathogenic organisms²⁹⁻³¹ and is necessary for bacterial penetration into host tissues³²⁻³³. Motile *Salmonella* have been shown to migrate away from functional vasculature in tumors, where they form colonies and delay tumor growth³⁴. The mechanisms that enable bacteria to initially cross the endothelium are unknown³⁵, but several experiments have demonstrated that bacteria colonize tumor interstitium outside blood vessels³⁵⁻³⁶. Colonization and spreading have also been studied in porous media. These systems are analogous to tumors where the walls of the medium correspond to cell membranes in the tissue. In porous media, motile bacteria penetrate better than non motile bacteria³⁷ and differences in motilities between different strains give rise to different diffusive behaviors³⁸. Reports about the role of motility in bacterial penetration and accumulation in tumors, however, have been contradictory. It has been shown for some bacterial strains that the extent of bacterial accumulation in tumors is independent of motility³⁹⁻⁴⁰. Some non-motile bacteria have been shown to have better transport properties through porous surfaces than motile bacteria⁴¹, possibly because of lesser probability of collision with pore surfaces⁴¹⁻⁴². Bacteria have also

been shown to penetrate into narrow constrictions, independent of motility, by means of growth and division alone⁴³.

Bacteria naturally have a therapeutic effect on tumors. Native toxicity of bacteria regresses tumors^{8, 15, 19–20, 44} and bacteria have also been used to express anticancer agents^{45–47}. Native toxicity has been attributed to competition for nutrients and sensitization of the host immune system⁴⁸. At low densities, invasive *Salmonella* have no intrinsic toxicity to tumor cells in vitro⁴⁹. However, *Salmonella* colonization in tumors causes apoptosis both in vitro⁶ and in vivo^{8, 34}.

To measure the spatiotemporal dynamics of bacterial accumulation and induced apoptosis, we developed an in vitro model of continuously perfused tumor tissue⁵⁰. We hypothesized that 1) *strains with higher motility have enhanced penetration and colonization*, and 2) *the location of bacterial colonization within tissue corresponds to the location of apoptosis*. To test these hypotheses, the motility of four strains, *Salmonella* (SL1344 and VNP20009) and *E.coli* (K12 and DH5 α), was measured in aqueous solution. The four strains were introduced into tissue and the location of colonization and induced apoptosis was measured over time with fluorescence microscopy. A mathematical model was used to determine the relative contribution of dispersion and growth, and predict behavior around blood vessels in tumors. Understanding the role of bacterial motility in tumor penetration, spreading, and apoptosis induction will be critical for designing effective bacterial therapies.

Results

Quantification of bacterial motility

Bacterial motility was quantified by time-lapse imaging of cells suspended in DMEM (Fig. 1A). The two *Salmonella* strains, SL1344 and VNP20009, were more motile ($P < 10^{-4}$) than the two *E. coli* strains, K12 and DH5 α (Fig. 1A,B). The average velocity of *Salmonella* was 14.5 ± 0.9 times that of *E. coli*. Among the four bacterial strains tested, average velocity decreased in the order: SL1344, VNP20009, K12 and DH5 α (Fig. 1B). The distribution of velocities also differed between the strains. SL1344 and VNP20009 had bimodal distributions with a fraction of their population being highly motile (Fig. 1C). The maximum velocity of SL1344, 26.5 $\mu\text{m/s}$, was higher than that of VNP20009, 23.6 $\mu\text{m/s}$ (Fig. 1C). The fraction of SL1344 population that was highly motile was greater ($P < 0.05$) than VNP20009 and the fraction that was minimally motile was lower ($P < 0.05$) than VNP20009 (Fig. 1D). The entire population (100%) of the two *E. coli* strains K12 and DH5 α was minimally motile (Fig. 1D) and this fraction was greater ($P < 0.05$) than the fractions of minimally motile *Salmonella* strains (Fig. 1D). The distribution of velocities for *E. coli* strains was unimodal (Fig. 1E) and the average velocity of K12 was higher ($P < 10^{-3}$) than that of DH5 α . The fraction of K12 population that was minimally motile was higher ($P < 0.01$) than that of DH5 α (Fig. 1F). There was no difference in size between individual bacterial of the four strains.

Accumulation of different bacterial strains in tissue

The accumulation of bacterial strains in tissue was measured in an in vitro model that consisted of rectangular human colon carcinoma tissue subjected to continuous medium perfusion through a microfluidic channel along one of the edges (Fig. 2A). This model was specifically designed to mimic the delivery and systemic clearance of bacteria. 10^5 or 10^7 CFU/ml suspensions of bacteria, engineered to express ZsGreen for visualization, were introduced into flow channels and flushed after one hour. Time-lapse microscopy was used to monitor their tissue accumulation over 34 hours after flushing. All results were seen in multiple replicates ($n = 3$). At 10^5 CFU/ml inoculation, the accumulation of SL1344 in tissue increased after flushing, and was negligible in flow channels (Fig. 2B). After 10 hours, accumulation of SL1344 in tissue was significantly higher ($P < 0.05$) than in channels (Fig. 2B), confirming that systemic clearance of bacteria was successfully mimicked. The total amount of bacterial accumulation in tissue varied considerably between the four strains (Fig. 2A). When inoculated at 10^5 CFU/ml, only the two *Salmonella* strains SL1344 and VNP20009 had detectable colonization in tissues and there was no detectable colonization of the two *E. coli* strains K12 and DH5 α (Fig. 2A). Amongst the *Salmonella* strains, SL1344 exhibited higher accumulation in tissue compared to VNP20009, over the 30-hour observation period (Fig. 2A,C). At 30 hours, the accumulation of SL1344 was 15 ± 3.6 times greater ($P < 0.05$) than VNP20009 and at least 1000 times greater ($P < 0.05$) than both *E. coli* strains (Fig. 2E). The accumulation of VNP20009 was at least 80 times greater ($P < 0.05$) than both *E. coli* strains (Fig. 2E). The tissue accumulation of the four strains strongly correlated with motility ($R^2 = 99.27\%$; Fig. 2E).

A higher inoculation density (10^7 CFU/ml) was used to observe the accumulation of the two *E. coli* strains (Fig. 2D,F), because they did not accumulate at detectable levels at the lower inoculation density (Fig. 2B,C). Both strains showed detectable levels of accumulation at the higher inoculation density, and K12 accumulated more than DH5 α (Fig. 2B,D). At 30 hours, the accumulation of the more motile K12 strain was significantly higher ($P < 10^{-3}$) than the less motile DH5 α strain (Fig. 2F), in accordance with the motility-accumulation correlation at low inoculation densities (Fig. 2E).

Spatial distribution of bacterial accumulation

The four bacterial strains had different spatial distributions within tissue (Fig. 3A,B). Most notably, strains differed in their ability to penetrate tissue and colonize regions distant from the flow channels. The proximal region of tissue between normalized distances of 0.1 and 0.2 was designated as 'zone-1' and the distal region between 0.5 and 0.6 as 'zone-2'. Colonization initiated in zone-1 and progressed to zone-2 (Fig. 3C,D). SL1344 penetrated deeper into tissues compared to VNP20009 when inoculated at 10^5 CFU/ml (Fig. 3A,C). The two *E. coli* strains did not colonize at this seeding density. At 30 hours, the zone-1 and zone-2 accumulation of SL1344 was higher ($P < 0.05$) than other strains and the accumulation of VNP20009 was higher ($P < 0.05$) than the *E. coli* strains (Fig. 3E). The accumulation of SL1344 in zone-2 was not significantly less than in zone-1 (Fig. 3E). The zone-2 accumulation of VNP20009 was less ($P < 0.05$) than zone-1 (Fig. 3E). The two *E. coli* strains K12 and DH5 α exhibited different spatial distributions when inoculated at 10^7 CFU/ml (Fig. 3B,D). K12 penetrated deeper than DH5 α (Fig. 3B,D). At 34 hours, the zone-1 and zone-2

accumulation of K12 was higher ($P<0.05$) than that of DH5 α (Fig. 3F). The zone-2 accumulation of both these strains was less ($P<0.05$) than zone-1 (Fig. 3F). The penetration depth, defined as the distance where the density was 10% of its maximum, of SL1344 was greater ($P<0.05$) than all other strains (Fig. 3G). The penetration depths of VNP20009 and K12 were greater ($P<0.05$) than DH5 α (Fig. 3G).

Mathematical model for dispersion and growth of bacteria

A mathematical model was used to determine the relative contribution of dispersion and growth to the amount and location of bacterial accumulation in tissue. The model balanced accumulation with dispersion and growth:

$$\frac{\partial C_b}{\partial t} = D \frac{\partial^2 C_b}{\partial x^2} + \mu C_b \quad (1)$$

where C_b (cells/ μm^3) is the average bacterial concentration, D ($\mu\text{m}^2/\text{s}$) is the dispersion coefficient, μ (1/s) is the growth rate, and x (μm) is the distance from the edge of tissue. To account for the one-hour bacterial inoculation, concentration at the front edge of tissue was set to the inoculation density for the first one hour, and to zero after that. Bacteria that penetrated tissue in the first hour spread into the tissue and colonized. The model was solved to obtain spatiotemporal bacterial profiles, $C_b(x,t)$, for 40 hours after inoculation.

Experimental data was fit to the model to determine the values of parameters D and μ for each strain (Fig. 4A,B,C). The dispersion coefficient, D , was greater ($P<0.05$) for SL1344 than all other strains, and was lower ($P<0.05$; Fig. 4B) for DH5 α than all other strains. The growth rate, μ , of SL1344 was the highest ($P<0.05$), VNP20009 was greater ($P<0.05$) than the E.coli strains, and K12 was greater ($P<0.05$) than DH5 α (Fig. 4C). Experimentally obtained penetration depths for the four strains strongly correlated with dispersion coefficients ($R^2 = 94.69\%$; Fig. 4D). Similarly, the normalized bacterial accumulation at 30 hours had a strong correlation with growth rate ($R^2 = 99.90\%$; Fig. 4E). Average velocity in aqueous medium correlated with the dispersion coefficient in tissue ($R^2 = 70.70\%$; Fig. 4F). To demonstrate the relative contribution of parameters D and μ , 30-hour spatial profiles were generated for nominal parameters values, which were selected to generate profiles of similar magnitude to SL1344 experimental profiles. Based on these profiles, dispersion coefficient, D , had a strong effect on how deep bacteria penetrated into tissues, and growth rate, μ , had a strong effect on the maximum concentration of colonization (Fig. 4G,H). With increasing D , bacteria penetrated deeper (location of peaks; Fig. 4G) and did not affect maximum concentration (constant height of peaks; Fig. 4G). With increasing μ , the maximum concentration of colonized bacteria increased and did not affect location (Fig. 4H).

Apoptosis induction by bacteria

Apoptosis in tissue was detected by maintaining a red fluorescent dye that irreversibly binds to activated caspase-3, a marker of apoptotic cells, in all flow solutions. Bacterial accumulation caused apoptosis in the tumor tissue (Fig. 5). The extent of apoptosis in SL1344-treated tissue increased with increasing bacterial accumulation over the 30-hour observation period and decreased with time for control tissues (Fig. 5A,B). Overall bacterial

uptake in tissues correlated strongly ($R^2 = 98.60\%$) with the increase in apoptosis (Fig. 5B inset) over time. The increase in apoptosis in tissues varied with distance from the channel and was greater where there was more bacterial accumulation (Fig. 5C). At 30 hours, the spatial profile of apoptosis increase correlated strongly with the profile of bacterial accumulation ($R^2 = 92.90\%$; Fig. 5C inset). For controls, apoptosis decreased over entire tissues because of the favorable nutrient environments caused by continuous perfusion, except in the far distal regions (normalized distances > 0.8), where apoptosis increased marginally because of nutrient deprivation (Fig. 5C). Apoptosis in zone-1 of SL1344-treated tissues at 30 hours was more ($P < 0.05$) than in zone-2 (Fig. 5D). Overall, apoptosis in tissues increased only when and where bacteria colonized. At 10^5 CFU/ml inoculation, only the two *Salmonella* strains SL1344 and VNP20009 colonized tissues and increased apoptosis higher ($P < 0.01$) than controls (Fig. 5E). For the two *E.coli* strains that did not colonize, apoptosis decreased similar to controls, and was less ($P < 0.01$) than apoptosis caused by the *Salmonella* strains (Fig. 5E).

Discussion

The in vitro model used for these experiments mimics tumor regions adjacent to blood vessels. The delivery of bacteria was mimicked by flowing bacterial suspensions through the microfluidic channel and systemic clearance was mimicked by flushing with bacteria-free medium after one hour of treatment. Based on this arrangement, bacterial accumulation in the tissue model relates to the tumor colonizing ability of the bacterial strain, and colonization distant from flow channels relates to the ability of the strain to penetrate and distribute within tumors.

The higher accumulation of *Salmonella* compared to *E.coli* in tissue (Fig. 2B) suggests that *Salmonella* have better tumor colonizing and anticancer therapeutic abilities. A 15-fold higher accumulation of SL1344 compared to VNP20009 (Fig. 2C,E) shows that there is significant variability in tissue colonization between strains of the same species. Less accumulation of VNP20009 may be attributed to the loss of virulence caused by deletion of *msbB* and *purI* loci⁵¹. At the lower inoculation density (10^5 CFU/ml), only *Salmonella* accumulated in tissue and the accumulation of *E.coli* was undetectable (Fig. 3B,C). A 100-fold higher inoculation density was necessary to observe accumulation of *E.coli* (Fig. 2B). This suggests that the minimum effective dosage for *E.coli*-based anticancer therapies would be significantly higher than *Salmonella*. High bacterial doses can increase systemic toxicity and could render *E.coli*-based therapies ineffective. Minimal accumulation of DH5 α , even at the high inoculation density (Fig. 2B,D), further suggests that DH5 α has limited therapeutic ability.

Apoptosis measurements (Fig. 5) show that bacteria have a natural therapeutic ability against tumors. There was no noticeable time lag between bacterial tissue accumulation and increase in apoptosis (Fig. 5A,B,C), indicating that the time scale of apoptosis induction was short. Because these experiments were performed in vitro in the absence of an active immune system, apoptosis was not caused by immune sensitization¹⁶. *Salmonella* that invade mammalian cells are not intrinsically toxic to cancer cells at low densities⁴⁹. These results suggest that induced apoptosis may be a result of nutrient deprivation caused by bacterial

colonization^{19–20, 44}. At high bacterial densities, however, invasive *Salmonella* may have a deleterious effect on cancer cells, which could be another mechanism of apoptosis induction.

Comparison of the penetration of *Salmonella* and *E.coli* (Fig. 3A) shows that motility is essential for effective distribution in tumors. Deep penetration of therapeutic agents is important to overcome the limitations of passive molecules, which cannot treat tumor regions far from vasculature. Highly motile strains are necessary for the treatment of advanced primary tumor masses, which are sparsely vascularized, because colonization at large distances from blood vessels is necessary for eradication. Less motile strains will colonize only in the vicinity of blood vessels and their therapeutic ability will be limited to smaller, early stage tumor masses. Effective dispersion and penetration into tumors can be obtained by using faster bacterial populations, based on the positive correlation between velocity and dispersion (Fig. 4F). If all but highly motile ($>15 \mu\text{m/s}$) bacteria were eliminated from the population of VNP20009 used for these experiments, for example, the average velocity of the population would increase from $6.4 \mu\text{m/s}$ to $20.5 \mu\text{m/s}$. For this hypothetical strain, the tissue dispersion coefficient would increase from $0.114 \mu\text{m}^2/\text{s}$ to $0.674 \mu\text{m}^2/\text{s}$ (Fig. 4F), and the model predicts that these bacteria would penetrate deeper into tissue (Fig. 6A). Bacterial accumulation correlated strongly with apoptosis (Fig. 5C inset). Based on this correlation, deeper penetration of a highly motile population would cause apoptosis in tumor regions farther than $800 \mu\text{m}$ from vasculature (Fig. 6B). Without removing the less motile bacteria, the population would be ineffective at causing apoptosis in regions farther than $400 \mu\text{m}$ (Fig. 6B). VNP20009 has previously been tested in clinical trials but did not successfully reduce tumor burden²⁵. Based on these results, elimination of the less motile fraction, or selecting for the highly motile fraction of a VNP20009 population, would increase tumor penetration and improve clinical efficacy.

Bacterial accumulation in tumors is a result of the competing interactions of dispersion and growth within tissue. Dispersion increases the distance of bacteria from blood vessels. Growth increases bacterial concentration at the current location resulting in higher colonization densities. Based on a mathematical model of the relative contribution of these two phenomena (Eq. 1), higher growth rates can increase colonization densities but cannot lead to enhanced penetration and colonization in regions of tumors distant from vasculature (Fig. 4G,H). For deep penetration, high bacterial dispersion coefficients are necessary (Fig. 4G,H), which can be obtained by using populations with higher swimming velocities (Fig. 4F and 6A). Preferential bacterial growth within tumor microenvironments is therefore not sufficient to eradicate tumors; a combination of preferential growth and motility is necessary. Because *Salmonella* strains are significantly more motile than *E.coli*, the two species may adopt fundamentally different mechanisms for colonizing tumors. More motile *Salmonella* may actively penetrate into tumors and subsequently colonize in favorable environments (Fig. 6C). Less motile *E.coli*, having less ability for active penetration, may colonize near vessels (Fig. 6D). As they grow and divide, their density increases, and individual bacteria from these colonies slowly move down the concentration gradient away from vessels (Fig. 6D). This mechanism is supported by previous evidence of *E.coli* penetrating into narrow constrictions, independent of motility, by means of growth and division alone⁴³. Localized accumulation of less motile strains by this mechanism, however, will be limited by nutrient availability. Motility may therefore increase the uptake of bacteria

in tumor tissues by providing them access to new regions and sources of nutrients (Fig. 6A,C).

Conclusions

We have measured the effect of motility on the spatiotemporal dynamics of bacterial accumulation in tumor tissue. We employed an in vitro model that consists of continuously perfused solid tumor tissue that mimics the delivery and clearance of bacteria. *Salmonella* strains were significantly more motile than *E.coli* strains. At lower inoculation densities, only *Salmonella* strains colonized tumors; higher inoculation densities were necessary for the colonization of *E.coli* strains. The extent of bacterial colonization in tumors increased with increasing motility. Motility also affected the spatial distribution of bacterial accumulation within tissues and motile strains penetrated deeper. These results suggest that for efficient solid tumor therapies, highly motile strains should be selected. A mathematical model that described the relative contribution of bacterial dispersion and growth to the location and extent of bacterial accumulation in tumors showed that dispersion leads to deeper penetration into tumors and growth leads to increased bacterial densities. A combination of preferential growth within tumor microenvironments and motility is therefore essential for maximizing therapeutic efficacy. Bacterial accumulation caused apoptosis in tumors and the amount of induced apoptosis correlated strongly with bacterial density. These results demonstrate the importance of bacterial motility for tumor penetration, distribution and colonization, and suggest that motile *Salmonella* have higher therapeutic potential than less-motile *E.coli*.

Materials and Methods

Bacterial cultures

Salmonella strains SL1344 (*Salmonella Genetic Stock Center*, Alberta, Canada) and VNP20009 (*Vion Pharmaceuticals*, New Haven, CT), and *E.coli* strains K12 (MG1655; *ATCC*) and DH5 α (*Invitrogen*, Carlsbad, CA) were grown in Luria-Bertani medium (LB) and on LB-agar plates using standard bacterial culture protocols. SL1344 and K12 are wild-type strains, VNP20009 is a genetically modified non-virulent strain containing deletions in the *msbB* and *purI* loci, and DH5 α is a derivative of K12 that is optimized for molecular transformations. To enable visualization, all strains were transfected with a plasmid that constitutively expresses ZsGreen (*Clontech*, Mountain View, CA). Plasmids were transfected by electroporation with a Gene Pulser Xcell (*Bio-Rad*, Hercules, CA) system in 1 mm cuvettes using parameters 1.8kV, 25 μ F and 200 Ω .

To quantify bacterial motility, swimming velocities in mammalian cell culture medium were measured. Liquid cultures of bacteria were grown overnight and resuspended in Dulbecco's Modified Eagles Medium (DMEM; *Sigma-Aldrich*, St. Louis, MO). A 20 μ l droplet of the culture was placed on a glass slide and green fluorescence images were acquired every 0.53 or 0.62 seconds for 1 minute. To measure velocities, all bacteria in a frame (approximately 50) were tracked for 5 seconds, X and Y coordinates were noted at each time frame, and an average velocity was calculated. Bacteria with average velocities < 0.6 μ m/s were designated as non-motile, between 0.6 and 6 μ m/s as minimally motile, and above 15 μ m/s as highly

motile. Statistical comparison of mean velocities was made using the Kruskal-Wallis test to account for multimodal distributions.

Mammalian cultures

Human colorectal adenocarcinoma cells (LS174T) obtained from *ATCC* (Manassas, VA) were grown in T75 flasks in DMEM with low glucose (1 g/l), supplemented with 10% fetal bovine serum (FBS; *Sigma-Aldrich*). Multicellular tumor spheroids were formed by seeding 2.5×10^4 cells/ml on poly(2-hydroxyethyl methacrylate; *Sigma-Aldrich*) coated T25 flasks for 12–14 days. All cultures were maintained under 5% CO₂ and 100% humidity.

Microfluidic devices were used for continuous culture of LS174T tissue and measurement of the dynamics of bacterial tissue accumulation. We have previously measured bacterial motility and accumulation using tumor cylindroids^{5–6}. The present microfluidic device is an improvement over cylindroids because continuous medium perfusion prevents logarithmic growth in the culture medium and enables experiments longer than 20 hours. In addition, the perfusion of culture medium mimics bacterial delivery and clearance through the vascular system. Devices were fabricated using standard soft lithography techniques as described previously⁵⁰. Molds for devices were fabricated by contact soft lithography to produce positive relief SU-8 (*MicroChem*, Newton, MA) features over silicon wafers. Replicates were made using Sylgard® 184 Silicone Elastomer Kit (*Dow Corning*, Midland, MI). Inlets and outlets were punched with a 1.5 mm biopsy punch and the elastomer chips were bonded to glass slides after oxygen plasma treatment. Teflon tubing (0.032" ID) was attached to the inlets and outlets of the devices using barbed luer-lock connectors (*Qosina*, Edgewood, NY). We have previously shown that in the device there is no gap between the cells and the glass slide and that this interface does not affect molecular transport⁵⁰.

Spheroids were introduced into 1000µm × 300µm × 150 µm chambers on the devices to form cuboidal tissue as described previously⁵⁰ and were incubated for 24 hours at 37°C in a customized temperature controlled incubator built on a microscope. Humidity and pH inside devices were maintained by continuous perfusion of HEPES (25 mM) buffered DMEM at 3 µl/min. Ampicillin (100 µg/ml) was maintained in all flow solutions introduced into the device to maintain plasmids in transformed bacteria.

Bacterial treatment and apoptosis detection

Bacterial cultures were grown overnight in LB, centrifuged and resuspended in DMEM, diluted to a concentration of 10⁵ or 10⁷ CFU/ml, and introduced into devices at 3 µl/min. After 1 hour, devices were flushed with bacteria-free medium. Bacterial colonization was monitored for 34 hours post-inoculation. Inoculation densities of 10⁵ CFU/ml were used for all 4 strains and 10⁷ CFU/ml were used for the two *E. coli* strains. Controls were run without bacterial inoculation. At least 3 tissues were tested under each condition and statistical comparisons were made using Student's t-test. All error bars represent standard errors of the mean.

Apoptosis in tissues was detected by maintaining 0.4 µl/ml Red-DEVD-FMK fluorescent dye (CaspGLOW Red Active Caspase-3 staining kit; *BioVision*, Mountain View, CA) in all flow solutions. Red-DEVD-FMK is cell permeable, non-toxic, and irreversibly binds to

activated caspase-3, a marker of apoptotic cells. The dye was introduced after 24-hour incubation of tissues in devices, and 6 hours prior to bacterial inoculation to allow penetration of the dye into tissue.

Image acquisition and data analysis

All images were acquired using an *Olympus* (Center Valley, PA) IX71 inverted epifluorescent microscope equipped with Plan-APO 10× and SLCPlan 40× objectives. To capture an entire chamber of the microfluidic device (1000 μm $\times$ 300 μm), 2 images (867.15 μm $\times$ 660.68 μm each) obtained at 10× were tiled using a macro in IPLab (*BD Bioscience*, Rockville, MD). Transmitted light and green and red fluorescent images of chambers were captured at 1-hour intervals after bacterial inoculation. For quantification of bacterial motility, green fluorescence images of individual bacteria were obtained at 40×. Green and red images were captured using 470/40 nm and 546/10 nm excitation and 525/50 nm and 590 nm long pass emission filters (*Chroma*, Rockingham, VT), respectively.

Intensity values from green and red images were used to generate space and time profiles. A rectangular region of interest (ROI) incorporating each tissue was created at every time point. For each ROI, a background subtracted average intensity was calculated, and a linear average intensity profile was generated by averaging pixel intensities along successive widths of the ROI. Distances were normalized by the length of the ROI, which changed as the tissue grew. For analysis, the zero time point was set to be immediately after clearance of bacteria from channels. At 30 hours, the penetration depth of each bacterial strain was calculated for inoculation densities that resulted in detectable colonization, as the distance from the flow channel at which bacterial intensity decreased to 10% of its maximum. In cases where the intensity did not fall to 10%, the penetration depth was assumed to be greater than the length of the ROI. The entire procedure was automated using a customized script in MATLAB (*The MathWorks Inc*, Natick, MA).

Intensity values from green fluorescence images were calibrated to obtain bacterial densities within spheroids. For each bacterial strain, a suspension in DMEM with a known density was introduced into a device that did not contain tissue, and images were acquired before and after introduction of the suspension. A calibration was performed to relate fluorescence intensity to bacterial concentration. The OD600 to CFU/ml conversion factor was experimentally determined to be 5×10^8 by plating cultures of known density on LB agar plates and counting colonies. To calculate bacterial density per tissue mass, the density of tissue was assumed to be 1 g/ml. In cases where bacterial accumulation was undetectable, the bacterial density was assumed to be at or beneath the detection limit of the camera. To estimate the relative colonizing ability of each strain, a normalized dimensionless bacterial concentration was evaluated by dividing the bacterial density in tissue at 30 hours by the inoculation density.

Mathematical model of bacterial penetration and growth

The mathematical model of bacterial penetration and growth (Eq. 1) was based on a similar model developed previously in our laboratory⁵. The model was discretized using finite differences to obtain a set of algebraic equations and solved using Implicit Euler's method to

40 hours post inoculation. Gradients in bacterial concentration along the width of the chamber were assumed to be negligible. The initial concentration of bacteria in tissue was zero. An impermeable boundary was assumed at the rear end of the tissue. The concentration at the front edge was set to 10^5 or 10^7 CFU/ml for $t \leq 1$ hr and to zero for $t > 1$ hr to match experimental conditions. The size of the tissue ($L = 800\mu\text{m}$) was assumed to be constant because its rate of change was considerably slower than the rate of bacterial dispersion and growth.

To determine optimal values of D and μ , unconstrained nonlinear optimizations were run using the MATLAB function *fminsearch* to minimize the sum of squared errors between the simulated and experimental profiles at 30 or 34 hours. Parameters were fit to three independent experiments for each strain. Averaged optimized parameter values were used to generate optimum simulated profiles. 10^5 CFU/ml inoculation experiments were used for *Salmonella* strains and 10^7 CFU/ml experiments for *E.coli* strains. To demonstrate the relative contribution of parameters D and μ , 30-hour spatial profiles were generated for nominal parameters $D = 0.1 \mu\text{m}^2/\text{s}$, $\mu = 0.55, 0.57$ and 0.60 hr^{-1} , and for $\mu = 0.57 \text{ hr}^{-1}$, $D = 0.02, 0.07$ and $0.17 \mu\text{m}^2/\text{s}$.

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Figure 2. Accumulation of different bacterial strains in tumor tissue

A. Merged green fluorescence and transmitted light images of tissues at 30 hours. Scale bar is 500 μ m. Images of tissues were obtained over time, following 1-hour bacterial treatment and flushing. There was considerable difference in accumulation between strains. **B.** Accumulation in flow channels after 10 hours was significantly less (*, $P < 0.05$; $n = 3$) than tissue. **C,D.** Accumulation in tissue increased over time for 10^5 CFU/ml inoculation (**C**; $n = 3$) and for 10^7 CFU/ml inoculation of *E. coli* strains (**D**; $n = 3$). **E.** For 10^5 CFU/ml inoculation, accumulation at 30 hours correlated strongly ($R^2 = 99.27\%$) with velocity. Accumulation of SL1344 was greater (*, $P < 0.05$, $n = 3$) than all other strains and VNP20009 was greater (*, $P < 0.05$, $n = 3$) than *E. coli* strains. **F.** For 10^7 CFU/ml inoculation, K12 had higher (*, $P < 10^{-3}$) velocity (white bars) and greater (*, $P < 10^{-3}$, $n = 3$) accumulation (black bars) at 30 hours.

Figure 3. Spatial distribution of bacterial accumulation in tissue

A,B. Merged green fluorescence and transmitted light images of tissues treated with four bacterial strains at 10^5 CFU/ml at 15, 25 and 30 hours (A), and tissues treated with *E.coli* strains at 10^7 CFU/ml at 15, 25 and 34 hours (B). Scale bar is 500 μm. Color scales are provided to distinguish magnitudes of accumulation. At 10^5 CFU/ml, maximum density of SL1344 and VNP20009 was approximately 10^{10} and 10^9 CFU/g, respectively. At 10^7 CFU/ml, maximum density of K12 and DH5α was approximately 10^{10} and 10^8 CFU/g, respectively. **C,D.** 30-hour bacterial density profiles for SL1344 and VNP20009 at 10^5 CFU/ml (C; n=3) and K12 and DH5α at 10^7 CFU/ml (D, n=3). Magnitudes of densities differed considerably (y-axis scales). **E,F.** 30-hour zone-1 (black bars) and zone-2 (white bars) accumulation at 10^5 CFU/ml (E; n=3) and of *E.coli* strains at 10^7 CFU/ml (F; n=3). Zone-1 accumulation was significantly greater (*, $P < 0.05$) for all strains except SL1344. **G.** At 30 hours, penetration depth of SL1344 was greater (*, $P < 0.05$; n=3) than all other strains and of DH5α was less (*, $P < 0.05$; n=3) than all other strains.

Figure 4. Mathematical modeling of bacterial penetration and growth

A. 30-hour simulated and experimentally observed bacterial density profiles in tissue. Experimental data was fit to Eq. 1 to obtain optimized values of dispersion coefficient, D , and growth rate, μ , for each strain. Simulated profiles were generated for optimized parameter values. **B.** Dispersion coefficients of the four strains. SL1344 had the greatest (*, $P < 0.05$; $n = 3$) dispersion coefficient. **C.** Growth rates of the four strains. Growth rates decreased (*, $P < 0.05$; $n = 3$) progressively in the order SL1344, VNP20009, K12 and DH5α. **D, E, F.** Correlation between dispersion coefficient, D , and 30-hour penetration depth (D); growth rate, μ , and 30-hour normalized bacterial accumulation (E); and average velocity and dispersion coefficient, D (F). **G, H.** 30-hour bacterial density profiles generated for nominal values of dispersion coefficient, $D = 0.02, 0.07$ and $0.17 \mu\text{m}^2/\text{s}$, and growth rate, $\mu = 0.57 \text{ hr}^{-1}$ (G), and $D = 0.1 \mu\text{m}^2/\text{s}$, $\mu = 0.55, 0.57$ and 0.60 hr^{-1} , at 30 hours (H). Increasing D caused bacteria to penetrate deeper and increasing μ increased the density of accumulation.

Figure 5. Apoptosis induction in tissue

A. Merged green fluorescence and transmitted light images (top row), and merged red fluorescence and transmitted light images (bottom row) of SL1344-treated tissue. Green fluorescence corresponds to ZsGreen-expressing bacteria and red fluorescence corresponds to an active-caspase-3 marker, Red-DEVE-FMK. Scale bar is 500 μm . **B.** Normalized fluorescence intensities from red and green fluorescent images of SL1344-treated and control tissues ($n=3$). *Inset:* Data plotted as normalized increase in apoptosis intensity against bacterial intensity for treated tissue ($n=3$). Accumulation correlated strongly ($R^2 = 98.6\%$) with apoptosis over 30 hours. **C.** Spatial distribution of bacterial accumulation and apoptosis induction in SL1344-treated tissues and controls, at 30 hours ($n=3$). *Inset:* Data plotted as normalized increase in apoptosis intensity against bacterial intensity ($n=3$). At 30 hours, accumulation correlated strongly ($R^2 = 92.90\%$) with apoptosis. **D.** Apoptosis in zone-1 of SL1344-treated tissue at 30 hours was greater (*, $P<0.05$; $n=3$) than zone-2. **E.** Normalized increase in whole tissue apoptosis for control tissues and different bacterial treatments at 10^5 CFU/ml ($n=3$). Only *Salmonella* strains increased (*, $P<0.05$) apoptosis in tissue.

Figure 6. Bacterial penetration and spreading in tumors

A. Simulated 30-hour bacterial density distribution profiles for VNP20009, and a hypothetical strain that contains only the highly motile fraction of VNP20009. Elimination of less motile bacteria increased penetration. **B.** The hypothetical strain containing highly motile bacteria causes apoptosis farther than 800 μm from blood vessels. **C,D.** Proposed mechanism for penetration and accumulation of motile bacteria (**C**) and non-motile bacteria (**D**) in tumors. Motile bacteria are able to disperse, find favorable microenvironments, and colonize near and far from blood vessels. Non-motile bacteria colonize near blood vessels and slowly penetrate by growth and division.