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Persistent enhancement of bacterial motility increases tumor penetration

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Abstract

Motile bacteria can overcome the transport limitations that hinder many cancer therapies. Active bacteria can penetrate through tissue to deliver treatment to resistant tumor regions. Bacterial therapy has had limited success, however, because this motility is heterogeneous and within a population many individuals are non-motile. In human trials, heterogeneity led to poor dispersion and incomplete tumor colonization. To solve these problems, a swarm-plate selection method was developed to increase swimming velocity. Video microscopy was used to measure the velocity distribution of selected bacteria and a microfluidic tumor-on-a-chip device was used to measure penetration through tumor cell masses. Selection on swarm plates increased average velocity four fold, from 4.9 to 18.7 $\mu\text{m}/\text{sec}$ ($P < 0.05$) and decreased the number of non-motile individuals from 51 to 3% ($P < 0.05$). The selected phenotype was both robust and stable. Repeating the selection process consistently increased velocity and eliminated non-motile individuals. When selected strains were cryopreserved and subcultured for 30.1 doublings, the high-motility phenotype was preserved. In the microfluidic device, selected *Salmonella* penetrated deeper into cell masses than unselected controls. By ten hours after inoculation, control bacteria accumulated in the front 30% of cell masses, closest to the flow channel. In contrast, selected *Salmonella* accumulated in the back 30% of cell masses, farthest from the channel. Selection increased the average penetration distance from 150 to 400 μm ($P < 0.05$). This technique provides a simple and rapid method to generate high-motility *Salmonella* that have increased penetration and potential for greater tumor dispersion and clinical efficacy.

Introduction

Motile bacteria have the potential to overcome the diffusion restrictions that limit the efficacy of chemotherapeutic molecules. Motility is a key element of bacterial therapy because it enables penetration through tissue (Kasinskas and Forbes 2006; Kasinskas and Forbes 2007) and treatment of therapeutically resistant regions that are far from vasculature (Ganai et al. 2011; Zhang and Forbes 2015). Bacterial therapy is possible because therapeutic organisms, such as non-pathogenic *Salmonella*, specifically colonize tumors at ratios of 10,000:1 compared to healthy tissue (Forbes et al. 2003; Ganai et al. 2011). To

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improve efficacy, these bacteria have been engineered to express anticancer proteins that directly kill cancer cells (St Jean et al. 2014; Swofford et al. 2014) and induce apoptosis (Alexander et al. 2001; Ganai et al. 2009). *Salmonella* that are intrinsically motile have been shown to migrate away from vasculature in tumors, where they form colonies, induce apoptosis, and delay tumor growth (Ganai et al. 2011). This ability to migrate is essential for homogeneous colonization (Westphal et al. 2008) and effective therapy.

Bacterial therapy has had limited success, however, because of heterogeneous motility and tumor colonization. In tumors, several mechanisms control where bacteria colonize. There are two modes of bacterial motility, random dispersion and chemotaxis, and the local stroma composition affects penetration. *Salmonella* are attracted to sugars and amino acids released by dying cancer cells (Kasinskas and Forbes 2007). Metabolic gradients preferentially attract bacteria to the transition region between viable and necrotic regions in tumors (Ganai et al. 2011). Heterogeneous metabolic and stromal gradients would differentially affect bacterial colonization.

Bacteria also migrate through tissue via random dispersion, not directed by chemical gradients (Kasinskas and Forbes 2006). In tumors, less than 15% of bacteria actively penetrate tissue (Zhang and Forbes 2015). The remainder grew into colonies directly bordering blood vessels (Zhang and Forbes 2015). This ratio is similar to the percentage of actively motile bacteria in aqueous culture (Toley and Forbes 2012). The considerable fraction of non-motile individuals reduces the average velocity of the population (Toley and Forbes 2012). In tumor cell masses in a microfluidic device, more motile strains, e.g. *Salmonella enterica* SL1344, penetrate further than less motile strains, e.g. *E. coli* DH5 α (Toley and Forbes 2012). For these strains, there is a linear correlation between aqueous swimming velocity and depth of tumor penetration. A mathematical model generated to interpret these results suggested that deleting the non-motile fraction would increase penetration (Toley and Forbes 2012).

In human trials, poor penetration lead to heterogeneous and incomplete colonization (Toso et al. 2002). An attenuated *Salmonella* strain (VNP20009) was administered to 24 patients with metastatic melanoma in a phase I trial. Tumor colonization was not detected in many of the patients by needle biopsy. One tumor was excised that had apparently negative colonization, and was discovered to contain 11,000 CFU/g. This contradictory result suggests that bacteria were spread heterogeneously through the tissue and were missed by the aspiration needle. Increasing the number of motile bacteria and the average motility has the potential to increase penetration and solve the problems of heterogeneous and incomplete colonization.

To create a bacterial therapy with enhanced penetration, we developed a serial screening process to isolate highly motile *Salmonella*. We hypothesized that *eliminating the non-motile population would lead to accumulation in distal regions of tumors*. We tested this hypothesis by selecting for motile *Salmonella* with low agar swarm plates. Selection with this common technique (Wang and Wood 2011) has been shown to increase flagellation and virulence (Chelvam et al. 2014; Harshey and Matsuyama 1994; Kearns 2010). The aqueous motility distribution of selected *Salmonella* was determined by video microscopy and

automated particle tracking. Robustness of the method was tested by repeating the process with different parental strains and testing motility after expansion and cryopreservation. Penetration into tumor cell masses was tested by inoculation into an *in vitro* tumor-on-a-chip device. Bacterial accumulation and localization were quantified over time with fluorescence microscopy. Establishing a highly motile bacterial strain with increased tumor penetration should help eliminate heterogeneous colonization and increase the efficacy of bacterial therapy.

Materials & Methods

Bacterial strains and mammalian cell culture

Attenuated (VNP20009; *msbB*⁻, *purI*⁻, *xyl*⁻) and wild-type (SL1344) strains of *S. typhimurium* were transformed with plasmid pAS0213, which contains the gene for green fluorescent protein (*gfp*) under control of the constitutive *p(lac)* promoter. Cultures were maintained on LB-agar plates and in liquid LB containing 100 mg/ml ampicillin. Human LS174T colorectal adenocarcinoma cells were grown in Dulbecco's modified Eagle medium (DMEM) with 1 g/L glucose, supplemented with 10% fetal bovine serum (FBS) and buffered with 25mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Multicellular tumor spheroids were formed by seeding 2.5×10^4 cells·ml⁻¹ on poly2-hydroxyethyl-methacrylate-coated flasks for 12 days.

Motility selection on swarm plates

Motile *Salmonella* were separated from heterogeneous starting cultures using swarm plates (Figure 1A). Swarm plates were made with LB and 0.3% agar in 10 cm Petri dishes. Prior to selection, bacteria were grown to mid-exponential phase in liquid LB. A sample was diluted to 6×10^6 CFU·ml⁻¹ in 1:1 DMEM/PBS to measure the baseline velocity distribution, as described below.

For selection, the liquid culture was diluted to 2.4×10^8 CFU·ml⁻¹ in LB, and 10 µl was inoculated into the center of swarm plates. Following 36 hours of growth at room temperature, bacteria were extracted from the outermost ring (Figure 1A). Extracted bacteria were used for three purposes: velocity measurement, further selection, and testing of stability. The velocity of extracted bacteria was measured immediately after extraction. To measure velocity, extracted bacteria were suspended in 40 µl DMEM/PBS, and 10 µl was deposited on microscope slides and visualized, as described below. For subsequent selection, extracted bacteria were suspended in 40 µl LB, and 10 µl was inoculated on new swarm plates. These plates were incubated 36 hours at room temperature and the extraction process was repeated. To test stability, extracted bacteria were suspended in LB and frozen, as described below.

Measurement of aqueous velocity

To measure the velocity distribution of a bacterial population, microscope images of bacteria in liquid medium were acquired and analyzed. Aqueous motility was determined in mammalian cell media (HEPES buffered DMEM with 10% FBS) diluted with 50% PBS for comparison to motility in mammalian cell masses. Bacterial cultures (10 µl) were placed on

microscope slides and covered with coverslips. Green fluorescence images were acquired every 0.54 seconds for 27 seconds (50 images) at 40X on an inverted epifluorescent microscope (Olympus, Center Valley, PA) equipped with Plan-APO 10X and SLCPlan 40X objectives and IPLab software (BD Bioscience, Rockville, MD). Fluorescence images were acquired using 470/40 nm excitation and 525/50 nm long pass emission filters (Chroma, Rockingham, VT). Velocities of individual bacteria were determined by single-particle analysis of acquired image stacks using the TrackMate plugin (Fiji image processing package) in ImageJ (NIH Research Services Branch). Individual bacterial particles were identified by segmentation and the resultant binary images were filtered to remove noise. Trajectories were determined by assigning each particle an identity, measuring X and Y coordinates over time, and linking the particles. Particle tracks were visually adjusted for errors, including repeated spots and broken tracks. Average velocities were determined for each track. The threshold for non-motile bacteria was 2.5 $\mu\text{m}/\text{sec}$, which is one bacterial body-length per second.

Stability of highly motile phenotype

To test the stability of the selection method, selected bacteria were cryogenically preserved and regrown. After extraction from swarm plates, selected bacteria were suspended in 40 μL LB, 13.3 μL glycerol was added to make a 1:3 glycerol-LB solution, and cultures were stored at -80°C . To regrow bacteria, frozen solutions were inoculated onto LB-agar plates containing 100 mg/ml ampicillin and incubated overnight for 20 hours at 37°C . Individual colonies were picked and grown in liquid LB containing 100 mg/ml ampicillin for 2.75 hours to mid-exponential phase. Cultures were centrifuged and resuspended in 1:1 DMEM/PBS at a density of 6×10^8 CFU/ml. Velocity distributions were measured by depositing 10 μL droplets on glass slides and acquiring fluorescence images over time, as described above.

Measurement of bacterial penetration into tumor cell masses

Bacterial penetration into cell masses was measured in a tumor-on-a-chip microfluidic device (Figure 1B) that mimics tissue bordering blood vessels in tumors (Toley et al. 2011; Walsh et al. 2009). The device was formed by soft lithography as describe previously (Toley et al. 2011; Walsh et al. 2009). It contained six rectangular ($1000 \times 300 \times 150 \mu\text{m}$) chambers designed to retain multicellular tumor spheroids. Spheroids were introduced through the central channel and allowed to equilibrate at 37°C for 12 hours before bacterial injection. Temperature was controlled with an incubator enclosure around the microscope. Media was continuously perfused at 3 $\mu\text{L}/\text{min}$ through the central channel and along one side of each chamber (Figure 1B).

Prior to device inoculation, control and selected bacteria were grown on LB-agar plates overnight at 37°C . Selected bacteria were grown from preserved frozen stocks. Individual colonies were collected, grown to mid-exponential phase in liquid LB, centrifuged, and resuspended in HEPES-buffered DMEM with 10% FBS at a density of 10^5 CFU/ml. Bacteria-containing medium was administered to devices via the microfluidic channels at 3 $\mu\text{L}/\text{min}$ for one hour. After inoculation, devices were flushed with bacteria-free HEPES-

buffered DMEM to mimic systemic clearance. Ampicillin (100µg/ml) was added to all solutions to maintain GFP-expressing plasmids in transformed bacteria.

To measure bacterial accumulation and penetration, transmitted and fluorescence images were acquired every hour. Images of each chamber ($1000 \times 300 \mu\text{m}$) were composed of two microscope images ($867.15 \times 660.68 \mu\text{m}$ each), acquired at 10X magnification and tiled together. Bacterial density profiles were determined by averaging pixel intensities along successive widths of each cell mass at every time point (Toley and Forbes 2012) using an automated script in MATLAB (The MathWorks Inc., Natick, MA). An average background intensity was calculated from images acquired prior to bacterial inoculation, and subtracted from all profiles. Distances were normalized by the depth of the cell mass. A normalized location of *zero* was next to the flow channel, and value of *one* was farthest from channel. The proximal region was the 30% of the cell mass closest the flow channel, and the distal region was the farthest 30% from the channel. The zero time point was immediately after clearance of bacteria from the channel.

Statistical analysis

Results are presented as mean $\pm$ standard error of mean (SEM). Hypothesis testing was performed with analysis of variance (ANOVA) or Student's t-test. Differences were considered statistically significant when $P < 0.05$.

Results

Selection on swarm plates increased motility

Salmonella motility was increased by selection on LB-agar swarm plates (Figure 2). An inoculum of *Salmonella* in aqueous cultures (strain VNP20009) was placed at the center of the plates and incubated at 37°C. After 36 hours, the area of the bacterial mass expanded (Figure 2A, right). Bacteria were extracted from the outer ring of this mass and the velocity distribution was immediately measured. The extraction process was repeated with this population of selected bacteria. Selected cultures were transferred to aqueous medium, which was placed at the center of new plates, and were again selected from the outer ring after 36 hours.

The velocity distribution of the unselected control population and the selected populations was measured by video microscopy (Figure 2B). Velocity was measured by tracking the centers of individual bacteria over time. For each population, the velocities of at least 200 individual bacteria were measured (Figure 2C). Selection on swarm plates did not affect growth (Figure 2D). The growth rate of selected bacteria ($0.916 \pm 0.038 \text{ hr}^{-1}$) was not statistically different from the growth rate of control bacteria ($0.924 \pm 0.041 \text{ hr}^{-1}$).

After selection, the average motility increased 2.2 times ($P > 0.05$; Figure 2E). A second selection increased the average motility an additional 1.7 times ($P > 0.05$). Relative to unselected controls, successive selections increased the average velocity 4.0 fold ($P > 0.01$). The increase in average velocity was caused by elimination of non-motile bacteria from the population (Figure 2C, F). Over 60% of the control population had a velocity less than 5 µm/sec (Figure 2C). After each selection, the percentage of slow ($< 5 \mu\text{m/sec}$) bacteria

decreased significantly ($P < 0.01$; Figure 2C). This decrease was caused by elimination of the non-motile (velocity $< 2.5 \mu\text{m/sec}$) fraction (Figure 2F). After one selection, the percentage of non-motile bacteria decreased 3.4 fold. A second selection further decreased this percentage 10.2 fold ($P < 0.01$; Figure 2F). Overall, two selections almost completely eliminated non-motile bacteria, reducing their percentage of the population by 34.5 fold ($P < 0.05$; Figure 2F).

The decrease in non-motile bacteria was balanced by an increase in rapidly moving individuals (Figure 2C). A single selection significantly increased the percentage of bacteria with velocities between $15\text{--}20 \mu\text{m/sec}$ compared to controls ($P < 0.05$). After both one and two selections, the percentage of bacteria with velocities greater than $15 \mu\text{m/sec}$ increased significantly ($P < 0.05$). In the control populations, no bacteria were detected with velocities greater than $25 \mu\text{m/sec}$. In comparison, after two selections, more than 16% of the bacteria were faster than $25 \mu\text{m/sec}$. While most ($>69\%$) of the control population had a velocity less than $5 \mu\text{m/sec}$, most ($>83\%$) of the doubly-selected population had a velocity greater than $15 \mu\text{m/sec}$ (Figure 2C).

Conservation of motility enhancement

The effect of swarm-plate selection on motility was robust (Figure 3). Selection had a similar effect on the velocity distribution of a wild-type *Salmonella* strain (SL1344; Figure 3A) as on the distribution of VNP20009 (Figure 2C). One selection increased the average velocity 2.8 fold ($P < 0.05$) and two selections increased the velocity 3.1 fold ($P < 0.05$; Figure 3B). Similar to the nonpathogenic strain (Figure 2F), swarm-plate selection eliminated non-motile bacteria (Figure 3C). The percentage of non-motile bacteria (velocity $< 2.5 \mu\text{m/sec}$) decreased 7.8 fold after a single selection ($P < 0.01$).

No unselected SL1344 bacteria had velocities greater than $20 \mu\text{m/sec}$. In comparison, more than 30% of selected bacteria were faster than $20 \mu\text{m/sec}$. Prior to selection, more than 65% of the control population had a velocity less than $5 \mu\text{m/sec}$ (Figure 3A). After each selection, the percentage of slow bacteria decreased significantly ($P < 0.01$; Figure 3A). Selection also increased the percentage of rapidly moving individuals. The fastest observed velocity was $37.2 \mu\text{m/sec}$ for a selected bacterium. One selection increased the percentage of bacteria in the $20\text{--}25$ and $25\text{--}30 \mu\text{m/sec}$ velocity ranges ($P < 0.05$), and two selections increased the percentage of bacteria in the $15\text{--}20 \mu\text{m/sec}$ range ($P < 0.05$). These changes in motility were conserved after repeating the selection process two times for both strains. The increases in average velocity and decreases in the percentage of non-motile bacteria were similar after each selection.

Motility phenotype is stable

The increased-motility phenotype was maintained across multiple generations. To test the robustness of the selected bacteria, they were frozen and regrown in culture for 23 hours. At a growth rate of 0.917 hr^{-1} (Figure 2C), this time allowed for 30.1 doublings. After these generations, the selected bacteria stain retained the high velocity phenotype (Figure 4). The average velocity of the subcultured population was 3.8 fold greater than the control population ($P < 0.001$; Figure 4A).

The percentage of non-motile bacteria in the subcultured population was 15.5 fold less than in the control population ($P<0.05$; Figure 4C). The same stability was seen for wild-type *Salmonella*, strain SL1344 (Figure 4C–D). When selected bacteria were frozen and subcultured, the average velocity was equivalent to the velocity immediately after selection and 2.7 times greater than unselected controls ($P=0.05$; Figure 4C). Subculturing wild-type *Salmonella* did not affect the percentage of non-motile individuals, which remained at 4.7 times less than controls ($P<0.05$; Figure 4D). In addition to showing that the high-motility phenotype was maintained through multiple generations, these results show that the phenotype was retained after cryopreservation and revival.

Enhanced motility increased tumor penetration

Selected *Salmonella* penetrated deeper into tumor cell masses than unselected controls (Figure 5). Bacteria were administered to tumor-on-a-chip devices for one hour and were then flushed from the system. All accumulation resulted from bacteria that adhered during this treatment period, and then infiltrated into the cell masses. After eight hours, colonies of fluorescent bacteria were visible in all chambers (Figure 5A,B). In all control chambers, bacteria were located at the front of the cell masses, on the surface nearest to the flow channel (Figure 5A). In devices treated with selected *Salmonella*, all bacteria colonies were at the back of chambers, far from the flow channel (Figure 5B). To quantify the location of bacteria within the cell masses, fluorescence images were converted into spatiotemporal density profiles ($n=3$; Figure 5C, D). Bacterial density was quantified as a function of distance from the flow channel and normalized by the maximum density in all chambers and the size of each cell mass. Control bacteria accumulated at the front, and selected bacteria accumulated at the back of the cell masses. Between 8 and 15 hours, the density of control bacteria at every location in the front 40% of the cell masses significantly increased ($P<0.05$, Figure 5C). Over the same period, the density of selected bacteria increased significantly in every location at the rear 20% ($P<0.05$, Figure 5D).

With time, all colonies grew and selected bacteria penetrated further into cell masses (Figure 6). The average densities of selected and control bacteria increased at approximately the same rates and times (Figure 6A). In all chambers, few bacterial colonies were observed before seven hours, after which density increased linearly. The distinct localization patterns of selected and control bacteria were conserved for all chambers (Figure 6B). To compare bacterial strains, cell masses were divided into regions based on distance to the flow channel. The proximal region was the 30% of cell mass nearest the channel; the distal region was the 30% farthest from the channel; and the central region was between these two. At 15 hours, the density of selected bacteria in the proximal region was significantly less than controls ($P<0.001$, Figure 6B). Comparatively, in the distal region, the density of selected bacteria was significantly greater than controls ($P<0.05$; Figure 6B).

This localization was the result of increased penetration (Figure 6C). The average location of control bacteria ($\sim 150\ \mu\text{m}$) remained relatively constant from 7 to 15 hours. In comparison, the average location of selected bacteria almost doubled over this period. Before seven hours, density was too low to accurately determine average location. By 13

hours, the location of selected bacteria was significantly greater than control bacteria ($P<0.05$) and, on average, had penetrated almost 400 μm into cell masses.

Discussion

We have developed a method to extract and isolate motile bacteria from a population of *Salmonella*. The method establishes a means to reach therapeutically resistant regions of tumors. The method is simple and rapid. Strains are selected by extracting motile individuals from the outer edge of swarm plates. Selected strains have increased aqueous motility, and repeating the process further increases bacterial velocity. Resultant bacteria have a highly motile phenotype that is stable across multiple generations. Selected bacteria penetrate deeper into tumor cell masses than unselected controls. This increased penetration supports the predictions of a computational model previously generated in our laboratory (Toley and Forbes 2012). These results suggest that motility is a key mechanism controlling bacterial accumulation in tumors.

When introduced to tumor cell masses, selected bacteria accumulated in a distinctive pattern (Figure 5) that suggests that colonization has two discrete phases. Selected bacteria only colonized the distal end of chambers and not throughout the masses (Figure 5B,D). In contrast, control bacteria accumulated at the front end of cell masses and not at the back (Figure 5A,C). The timing of the emergence of these patterns suggests that bacteria first penetrated and then proliferated. At the earliest times that colonies were visible (8 hours; Figure 5B, top), selected bacteria were already located at the back of chambers. A likely explanation of this early colonization at the rear of chambers is chemotaxis toward necrotic material. Necrosis always forms at back end of chambers because of nutrients gradients away from the flow channel (Kasinskas et al. 2014; Walsh et al. 2009). In tumors, *Salmonella* are attracted to the molecules released by dying cells in necrosis (Ganai et al. 2011; Kasinskas and Forbes 2007). Because they were more motile, selected bacteria would have responded to these attractants by penetrating into the cell mass. Control bacteria would have sensed the attractants but would have not been able to respond. Once established in the front and rear of the chambers, control and selected bacteria, respectively, grew and expanded into surrounding regions.

The separation of penetration and growth in two temporally distinct phases will have important implications for use in the clinic. Because of the large non-motile fraction, control bacteria never reached the back region of the chambers (Figure 6B). In addition, selection did not affect growth. Selected bacteria had the same growth rate in aqueous media (Figures 2D) and in tumor cell masses (Figures 6A). Growth is necessary for bacterial targeting because it is the major mechanism that promotes colonization in tumors over other organs (Forbes et al. 2003; Ganai et al. 2011). The timing of penetration followed by growth will enable more effective colonization. In tumors *in vivo*, migration away from vasculature immediately after injection, could promote better dissemination throughout tissue.

The stability of the enhanced-motility strain is essential for use in the clinic and preclinical investigations in animal models. After selection, the motile phenotype was persistent for more than 30 generations (Figure 4). This persistence shows that swarm-plate selection did

not simply isolate motile individuals within the population, but caused a more permanent change. An alternate outcome of the swarm-plate selection process could have been a transient increase in motility. If individuals extracted from the outer edge of the plates did not pass the higher motility phenotype to their progeny, the selection would have to be performed before each administration. This would have been problematic because of small volumes extracted from swarm plates. Multi-generation stability will enable expansion to the quantities necessary for dosing without loss of motility. In addition, maintenance of the motile phenotype through freezing and long-term preservation (Figure 4) will enable comparison between strains amplified from identical stock cultures, and therefore more accurate experiments.

Increased motility will greatly enhance clinical utility. Eliminating non-motile bacteria will increase penetration into tumor tissue. *In vivo*, this deeper penetration would enable more effective treatment of therapeutically resistant tumor regions that are far from vasculature. Targeting colonization to these regions will create a therapeutic able to overcome the limits of current cancer therapies. The predominant therapeutic strategy to utilize tumor-penetrating bacteria would be expression of a cytotoxic protein (Ganai et al. 2009; St Jean et al. 2014). The expression of GFP (Figure 5) demonstrates that selected bacteria retain the ability to express recombinant proteins. Increased penetration will also reduce heterogeneous accumulation and increase overall density. Heterogeneous colonization was the primary cause of failure of bacterial therapy in clinical trials (Toley and Forbes 2012; Toso et al. 2002). Overall, increased motility and tumor penetration will increase the efficacy of *Salmonella* as a cancer therapeutic.

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Figure 1.

Swarm plate selection and tumor-on-a-chip device. A) Motile bacteria were selected by inoculating droplets of liquid culture at the center of swarm plates (left). After incubation, bacteria were extracted from the outer ring (right). B) Microfluidic device used to measure bacterial penetration. Tumor tissue was contained within six $1000 \times 300 \times 150 \mu\text{m}$ chambers, alternately staggered on either side of a central flow channel. The illustration shows the first three chambers.

Figure 2.

Selection of motile *Salmonella*. (A) Motile bacteria were selected on soft-agar swarm plates. A small ring (arrow) was present directly after inoculation (0 hours, left) that spread to the outer edge of the plate by 36 hours (right). Bacteria were extracted from this outer edge (arrow). (B) Fluorescence images of two bacteria (arrows) from control and selected populations at 0.54, 1.08, and 1.62 seconds. Motility was determined by measuring locations in sequential images. Scale bars are 50 μm . (C) Velocity distributions of three populations: unselected controls ($n=231$), bacteria after one selection ($n=214$), and bacteria after two

selections (n=247). One and two selections decreased the percentage in the 0–5 $\mu\text{m}/\text{sec}$ range (**, $P<0.01$), and increased the percentage in the 15–20 $\mu\text{m}/\text{sec}$ range (*, $P<0.05$). (D) The growth profiles of control and selected *Salmonella* in liquid medium were equivalent. (E) Average population velocities after one (*, $P<0.05$) and two (**, $P<0.01$) selections were greater than controls. (F) The percentage of nonmotile bacteria (velocity < 2.5 $\mu\text{m}/\text{sec}$) decreased after two selections compared to controls (*, $P<0.05$) and one selection (**, $P<0.01$).

Figure 3.

Selection of wild-type *Salmonella*. (A) Velocity distributions of *Salmonella*, strain SL1344, after one and two selections on swarm plates. Selection decreased the percentage of bacteria in the 0–5 $\mu\text{m}/\text{sec}$ range (**, $P < 0.01$), and increased the percentage in the 15–20, 20–25, and 25–30 $\mu\text{m}/\text{sec}$ ranges (*, $P < 0.05$). (B) Average aqueous velocities increased significantly after selection (*, $P < 0.05$). (C) The percentage of non-motile bacteria (velocity < 2.5 $\mu\text{m}/\text{sec}$) decreased after selection (*, $P < 0.05$).

Figure 4.

Motile phenotype was preserved after cryopreservation and regrowth. (A) Average velocities of subcultured *Salmonella*, strain VNP20009, were at least as fast as the population after one selection (*, $P < 0.05$) and significantly greater than the control population (***, $P < 0.001$). (B) The percentage of non-motile individual bacteria was at least as small as after one selection (***, $P < 0.001$) and significantly less than the control population (*, $P < 0.05$). (C,D) Average velocities (C) and percentages of non-motile individual (D) of subcultured *Salmonella*, strain SL1344, were comparable to the population after one selection and significantly different from controls (*, $P < 0.05$).

Figure 5.

Selection increased *Salmonella* penetration into tumor tissue. (A, B) Merged fluorescent and transmitted microscopy images of control (A) and selected (B) *Salmonella* (green) in tumor tissue (grey). Images were acquired at 8, 10, and 15 hours after administration of 105 CFU/mL of bacteria for one hour and flushing with fresh media. Scale bars are 200 μ m. (C, D) Bacterial density profiles ($n=3$) as a function of normalized distance into tissue at 8, 10 and 15 hours, for control (C) and selected (D) *Salmonella*. For control bacteria, the density in the front 40% of the tissue significantly increased between 8 and 15 hours (*, $P<0.05$). For selected bacteria, the density in the rear 20% of the tissue significantly increased between 8 and 15 hours (*, $P<0.05$).

Figure 6.

Bacterial growth and penetration in tumor tissue. (A) Total bacterial density in tissue, determined from fluorescence images, increased at similar rates for control and selected *Salmonella*. (B) At 15 hours, the density of selected bacteria in the proximal region was significantly less than control bacteria (***, $P < 0.001$). In the distal region, the density of selected bacteria was significantly greater than controls (*, $P < 0.05$). The proximal and distal regions are defined as the 30% of tissue closest to the channel and the 30% of tissue farthest from the channel, respectively. (C) Average location of bacteria within tumor tissue relative

the edge bordering the flow channel. With time, selected bacteria penetrated deeper into tissue. At 13 and 15 hours, the average penetration depth of selected bacteria was significantly greater than control bacteria (*, $P < 0.05$).

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