Phenotypic Evolution of Therapeutic *Salmonella enterica* Serovar Typhimurium after Invasion of TRAMP Mouse Prostate Tumor

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ABSTRACT ***Salmonella*** **has been of interest in cancer research due to its intrinsic ability to selectively target and colonize within tumors, leading to tumor cell death. Current research indicates promising use of *Salmonella* in regular administrations to remove tumors in mouse models while minimizing toxic side effects. However, selection of mutants during such long-term tumor colonization is a safety concern, and understanding selection of certain phenotypes within a tumor is an important consideration in predicting the long-term success of bacterium-based cancer treatment strategies. Thus, we have made an initial examination of selected phenotypes in a therapeutic *Salmonella enterica* serovar Typhimurium population developed from an archival wild-type LT2 strain and intraperitoneally injected into a 6-month-old TRAMP (transgenic adenocarcinoma of mouse prostate) mouse. We compared the original injected strain to isolates recovered from prostate tumors and those recovered from the spleen and liver of non-tumor-bearing TRAMP mice through phenotypic assessments of bacteriophage susceptibility, motility, growth rates, morphology, and metabolic activity. Tumor isolate traits, particularly the loss of wild-type motility and flagella, reflect the selective pressure of the tumor, while the maintenance of bacteriophage resistance indicates no active selection to remove this robust trait. We posit that the *Salmonella* population adopts certain strategies to minimize energy consumption and maximize survival and proliferation once within the tumor. We find these insights to be nonnegligible considerations in the development of cancer therapies involving bacteria and suggest further examinations into the evolution of therapeutic strains during passage through tumors.**

IMPORTANCE ***Salmonella*** **is of interest in cancer research due to its intrinsic abilities to selectively target, colonize, and replicate within tumors, leading to tumor cell death. However, mutation of strains during long-term colonization within tumors is a safety concern, and understanding their evolution within a tumor is an important consideration in predicting the long-term success of bacterium-based cancer treatment strategies. Thus, we have made an initial examination of phenotypically diverse *Salmonella* colonies recovered from a therapeutic *Salmonella* strain that we developed and injected into prostate tumor-bearing mice. We compared the bacteriophage susceptibility, motility, growth rates, morphology, and metabolic activity of the original therapeutic strain to those of strains recovered from prostate tumors of tumor-bearing mice and the liver and spleen of non-tumor-bearing mice. Our results suggest that the *Salmonella* population adopts certain strategies to minimize energy consumption and maximize survival and proliferation once within the tumor, leading to phenotypic changes in the strain.**

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Current bacterium-based cancer therapy research is exploring the use of *Salmonella* bacteria which, when injected into tumor-bearing models, preferentially invade and replicate within tumor tissues, subsequently inhibiting their growth or destroying them entirely (1, 2). Importantly, these bacteria can penetrate deep within a tumor and, as facultative anaerobes, thrive in both hypoxic and oxygen-containing areas in the tumor environment. The development of a successful bacteriotherapy could lead to cancer treatments with high tumor-targeting specificity and precision, avoiding the often negative side effects of nonspecific cancer treatments resulting from destruction of healthy cells and tissue. *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) has proven to be a promising cancer therapeutic candidate due to its tumor-targeting and killing abilities (2, 3). Researchers have developed *S.* Typhimurium strains with reduced toxicity to reduce or eliminate the septic side effects often experienced in several animal hosts; one of these strains successfully colonized nonresponsive tumors in phase I human clinical trials (4). Despite the lack of adequate tumor regression observed in clinical trials for reasons outlined in published reports (4, 5), these findings have demonstrated the potential use of bacteria in cancer therapy and its short-term safety in humans, and *S.* Typhimurium remains an

TABLE 1 *S.* Typhimurium strains and phage isolates used in this study[a]

CRC stock no.	Strain or phage information and history	Ampicillin resistance
LT2	Wild-type *S.* Typhimurium strain, characterized in reference 29	No
1674	Derived from LT2 strain; *hisD2550 rpoS*; characterized further in reference 30	No
2631	Original therapeutic *S.* Typhimurium strain injected into TRAMP mice; CRC1674 *aroA*::Tn*10* Δ*rfaH*::pKD4 Δ*thyA*::pKD4, where CRC1674 is LT2 *hisD2550* (archived 1958, "resuscitated" 1999)	No
2636	CRC2631 that contains pRSET-mCherry plasmid (*bla*, mCherry) for fluorescence studies; characterized further in reference 11	Yes
2652	*S.* Typhimurium recovered from prostate tumor of tumor-bearing TRAMP mouse 3 days after injection with 2636	Yes
2653	*S.* Typhimurium recovered from prostate tumor of tumor-bearing TRAMP mouse 3 days after injection with 2636	Yes
2654	*S.* Typhimurium recovered from prostate tumor of tumor-bearing TRAMP mouse 3 days after injection with 2636	No
2835	*S.* Typhimurium recovered from liver of non-tumor-bearing TRAMP mouse 21 h after injection with 2636	Yes
2836	*S.* Typhimurium recovered from liver of non-tumor-bearing TRAMP mouse 21 h after injection with 2636	Yes
2837	*S.* Typhimurium recovered from spleen of non-tumor-bearing TRAMP mouse 21 h after injection with 2636	Yes
2838	*S.* Typhimurium recovered from spleen of non-tumor-bearing TRAMP mouse 21 h after injection with 2636	Yes
2754	Phage-free *S.* Typhimurium strain received from A. Segall; SDT2739 *S. enterica* serovar Typhimurium LT2MA8507 (Gifsy-1$^-$ Gifsy-2$^-$ Fels-2$^-$) ΔFels-1::*frt*; characterized further in reference 31	No
ES18	*S.* Typhimurium-specific bacteriophage characterized in reference 32	
O-1	*S.* Typhimurium-specific bacteriophage characterized in reference 32	
P22	*S.* Typhimurium-specific bacteriophage characterized in reference 32	
P162	Lilleengen bacteriophage (lyophilized); STM-30; characterized in reference 33	
P165	Lilleengen bacteriophage (lyophilized); STM-31; characterized in reference 33	
P282	Fels-1, Gifsy-2 supernatant collected by CRC in 2006	
P283	Fels-2, Gifsy-2 supernatant collected by CRC in 2009	

[a] Isolates 2652 to 2653 were recovered from the same mouse; isolates 2835 to 2838 also were recovered from the same mouse.

excellent candidate for cancer therapy either by itself or as a delivery vector (6–8) due to its extreme tumor-homing abilities even in advanced metastases (3).

Research is now shifting to implementing regular administrations of low *Salmonella* doses to effectively eliminate tumors in mouse models while minimizing the incidence of toxic side effects (3, 9, 10). Investigators at the Cancer Research Center (CRC; Columbia, MO) have engineered therapeutic strain CRC2631 (Table 1), derived from archival, wild-type *S.* Typhimurium LT2 kept in sealed agar stabs for over 40 years. Genetic manipulation using phage transduction and recombineering was employed to minimize the septic side effects of wild-type *Salmonella* strains while preserving their tumor-targeting and killing capabilities. A strain that preferentially targeted *tr*ansgenic *a*denocarcinoma of *m*ouse *p*rostate (TRAMP) mouse prostate tumors rather than normal *Salmonella* reservoirs of liver and spleen at ratios of 1,000:1 during a 24-h incubation period was isolated (11). Initial low-dosage studies established that weekly, low-dose injections of CRC2631 do not produce ill effects in immunocompetent mice and delay the mortality of TRAMP mice (R. Kazmierczak and A. Eisenstark, unpublished data). While clinical trials and murine research address toxicity and extension of survival, an evaluation of the mutability of therapeutic strains during colonization as a consideration for clinical safety and maintaining clinical effects has yet to be explored in research. Here, we report our phenotypic comparative assessments of the therapeutic strain (CRC2636, a derivative of CRC2631 that expresses red fluorescent tracer protein) before and after recovery either from the prostate tumor of tumor-bearing mice or from the liver and spleen of non-tumor-bearing mice as it affords insight into the evolutionary process of therapeutic *S.* Typhimurium in murine prostate tumors, the specificity of the tumor's selective pressures, and the safety of the long-term use of the therapeutic strain.

RESULTS

Therapeutic strains maintain bacteriophage resistance during passage through prostate tumor. With the exception of CRC2652 sensitivity to phage P283, the *Salmonella* isolates recovered from

TABLE 2 Bacteriophage susceptibility of strains

Isolate source	CRC stock no.	Reaction of strain[a]: ES-18	O-1	P22	P162	P165	P282	P283
	LT2	+	+	+	+	+	+	+
	1674	+	+	+	+	+	+	+
	2636	+	−	−	−	−	−	−
Prostate tumor	2652	+	−	−	−	−	−	P
	2653	+	−	−	−	−	−	−
	2654	+	−	−	−	−	−	−
Liver isolate (nontumor)	2835	+	−	−	−	−	P	P
	2836	+	−	−	−	−	P	P
Spleen isolate (nontumor)	2837	+	−	−	−	−	P	P
	2838	+	−	−	−	−	P	P

[a] +, complete clearing; −, no lysis; P, partial or faint lysis; $n = 3$ trials.

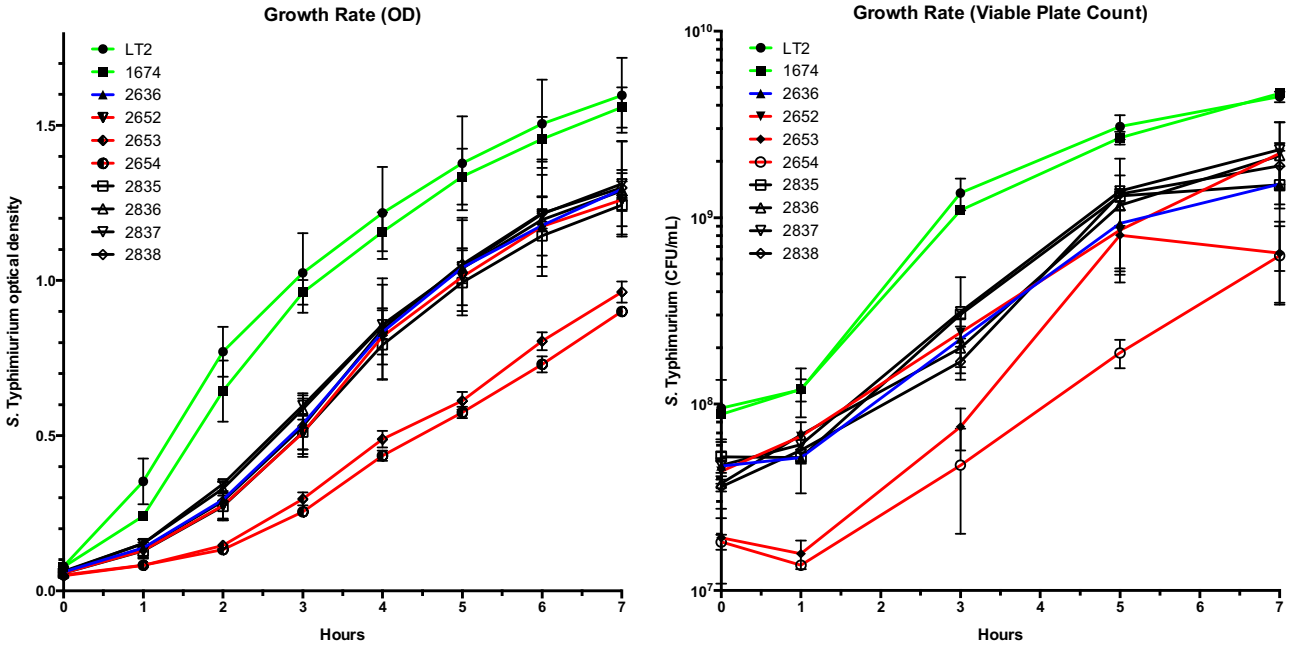

FIG 1 Assessment of strain growth rate averages via optical density or viable plate count versus incubation time. Overnight cultures were diluted to an OD of 0.05 for initial spectrophotometer readings. Optical density readings were performed every hour for 7 h, and culture dilutions followed by viable plate counting were performed every other hour for 7 h. Growth curves for strains are colored as follows: parental strains (LT2 and CRC1674), green; injection strain (CRC2636), blue; tumor-recovered strains (CRC2652, CRC2653, and CRC2654), red; strains recovered from liver or spleen (CRC2835, CRC2836, CRC2837, and CRC2838), black.

the prostate tumor maintain the phage resistance profile observed in the original, injected, therapeutic strain CRC2636 (Table 2). However, strains recovered from non-tumor-bearing mice exhibited a slight loss of phage resistance and were slightly susceptible to P282 and P283. Preliminary assays with 2 additional strains from healthy mice showed the same patterns as those with CRC2835 and CRC2837 (E. Choe, unpublished data).

Isolates recovered from tumors exhibit lower growth rates and delayed swarming phenotypes. Figure 1 illustrates the phenotypic difference in average growth rates—as measured by turbidity levels over time and viable plate counts—revealed by a comparison of tumor-bearing isolates and non-tumor-bearing isolates. Compared to the original injected parent strain CRC2636 and isolates from non-tumor-bearing mice, two of the three isolates recovered after passage through prostate tumors exhibited slower-changing optical density (OD) during the first 7 h of growth. One tumor-bearing isolate grew similarly to the CRC2636 parent strain. Isolates from non-tumor-bearing mice demonstrated more rapid growth than did the parent strain. However, turbidity reached comparable levels after overnight growth, though liver and spleen isolate cultures continued to have slightly higher turbidity. Similarly, a stark difference in motility ring development was observed (Table 3), as swarming in tumor isolates was delayed by as much as 48 h before the expansion of the motility ring began (though colonization and growth could be observed in the area of inoculation after the first day). However, as with the growth curve assessments, once the swarming began, the strains rapidly overtook the dish. Isolates recovered from healthy mice appeared to grow more rapidly than the original injected strain and to swarm on a level comparable to, if not slightly lower than, that of the original strain.

Isolate population recovered from tumor exhibits loss of flagella. Microscopy revealed that all examined isolates recovered from tumor-bearing mice lacked flagella, unlike the original therapeutic strain and most of the isolates from healthy mice (Fig. 2). When tested on motility agar, all strains were motile (Table 3). Tumor isolates also appeared to exhibit greater size variability between strains.

Isolates recovered from tumors exhibit varied carbon-containing substrate utilization and loss of sucrose metabolism. Incubation in ES MicroPlate (Biolog) plates revealed various metabolic profiles of substrate utilization, including loss and gain of utilization by both tumor isolates and healthy isolates compared to the original strain (Table 4). Of note are the loss in sucrose

TABLE 3 Average swarming diameter of strains on motility agar

Isolate source	CRC stock no.	Diam score at time[a]: 24 h	48 h	72 h
	LT2	7	33	+
	1674	7	+	+
	2636	2	32	+
Prostate tumor	2652	2	19	33
	2653	2	10	23
	2654	1	12	23
Liver isolate (nontumor)	2835	5	+	+
	2836	4	33	+
Spleen isolate (nontumor)	2837	4	32	+
	2838	4	33	+

[a] Swarming diameters were assigned a score designating a factor by which the smallest diameter measurement taken at 24 h (strain 2654) needed to be multiplied to reach the average swarming diameter of the given strain ($n = 3$). + designates overgrowth of the agar plate (a score of 34), after which measurements could not be taken.

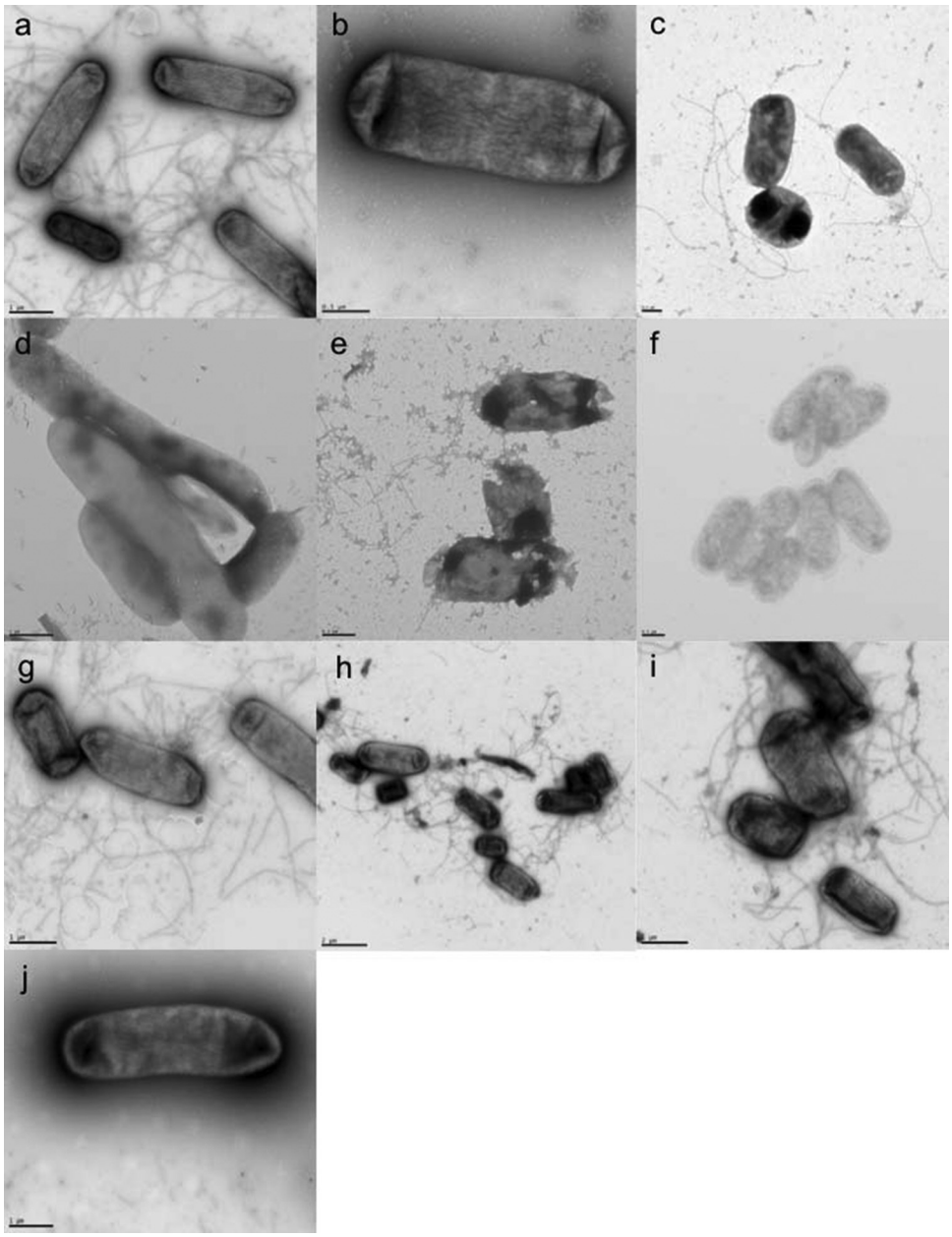

FIG 2 *Salmonella* isolate morphology. Isolates were recovered from the following environments: liquid culture (a to c), tumor (d to f), liver (g and h), and spleen (i and j). Strains were viewed using transmission electron microscopy under negative staining with 4% uranyl acetate on carbon-coated copper grids and included LT2 (bar, 1 μm) (a), CRC1674 (bar, 0.5 μm) (b), CRC2636 (bar, 0.5 μm) (c), CRC2652 (bar, 1 μm) (d), CRC2653 (bar, 0.5 μm) (e), CRC2654 (bar, 0.5 μm) (f), CRC2835 (bar, 1 μm) (g), CRC2836 (bar, 2 μm) (h), CRC2837 (bar, 1 μm) (i), and CRC2838 (bar, 1 μm) (j).

utilization by CRC2653 and CRC2654 (tumor isolates) and the overall variability in substrate utilization among tumor-bearing isolates.

DISCUSSION

This study seeks to begin a dialog on the selective environments of tumors in order to improve therapeutic bacteria, ensure patient safety during clinical use, and maintain therapeutic efficacy. We sought to discover what traits a tumor environment selects for or selects out after successful invasion by a large population of therapeutic bacterial cells. The initial isolates recovered from the prostate tumor maintained their bacteriophage resistance, while those from the liver and spleen lost their resistance to some phage. We find this to be a promising attribute of our therapeutic strain, as it illustrates not only its phenotypic robustness under physiological conditions in the tumor but also its potential compatibility with current therapeutic approaches that incorporate bacteriophages for their antitumor usage or for their ability to display peptides for anticancer use (12). Our therapeutic strain CRC2631 includes the deletion of *rfaH*, which changes the nature of the cell membrane by reducing the amount of available wild-type lipopolysaccharide (LPS) and dramatically reducing phage susceptibility, as many

TABLE 4 Variation in carbon-containing substrate utilization by isolates

Typing	Substrate	Utilization by strain[a]:					
		2636	2652 (tumor isolate)	2653 (tumor isolate)	2654 (tumor isolate)	2835 (liver isolate)	2837 (spleen isolate)
Salmonella	Sucrose	+	+	−	−	+	+
	Adonitol	−	+	−	+	−	−
	Glycolic acid	+	+	+	+	−	−
	Glyoxylic acid	+	+	+	+	−	−
	Phenylethyl amine	+	+	−	−	−	−
E. coli	Citric acid	+	+	+	+	−	−
	m-Inositol	−	−	−	−	+	+
	D-Threonine	−	+	−	−	+	+
	1,2-Propanediol	−	+	+	+	−	−
	m-Tararic acid	+	+	−	−	−	−
	Tricarballylic acid	+	+	−	−	+	+
	Tyramine	−	−	−	−	−	−
None	Formic acid	+	+	−	−	+	+
	Acetic acid	+	+	−	+	−	−
	Acetoacetic acid	+	−	+	−	−	−
	Methyl pyruvate	+	+	+	+	−	−
	2-Amino ethanol	+	+	−	−	+	+

[a] Data were not collected for LT2, 1674, 2836, and 2838.

phage bind to bacterial LPS before infection (13). The maintenance of phage resistance in tumor-bearing isolates suggests conservation of the *rfaH* deletion, and PCR of the *rfaH* gene performed on all strains confirmed conservation of this deletion among all strains (R. Kazmierczak, unpublished data). The slight susceptibility of non-tumor-passaged strains suggests that passage through healthy mice is affecting phage susceptibility independently of *rfaH*, which could be confirmed via silver staining to determine potential differences in LPS profiles.

More intriguing is the loss of flagellar structures and overall decreased motility and proliferation rates of isolates recovered from tumor-bearing mice. Flagella and swarming are considered essential for bacterial signaling systems and efficient infection of hosts (14, 15). However, we observed that tumor isolates exhibited a slime production on motility plates even when motility rings were unapparent. We attribute this slime to production of acidic polysaccharides, which act as biosurfactants to facilitate movement (16) and may serve a role in cell-to-cell communication (17, 18). With *S.* Typhimurium being a Gram-negative bacterium, it is possible that the tumor-bearing mouse isolates are expressing signaling molecules (15). Cells in these strains may wait to swarm or even proliferate until a quorum signal is sensed by the population—an adaptation to the volatile and nutrient-changing environment of the tumor. If quorum sensing, these bacteria would still be able to create biofilm formations on the tumor cell surfaces despite their lack of observable flagella (19), as they are still motile. It is important to note that *Salmonella* bacteria need not be motile in order to be virulent (20); however, while the tumor isolates lacked visible flagella, they were able to move on motility agar, albeit they were slow to do so. Alternatively, successful motility after a lag period coupled with high slime production suggests activation of the Rcs phosphorelay signaling system that inhibits flagellar synthesis while simultaneously increasing capsular polysaccharide production (18). In order to prevent low growth rates from confounding the motility assessment, we observed swarming diameters of cultures that had been taken from the actively migrating periphery of motility plates from the previous day. Thus, we observe lower motility and overall lower growth rates of tumor-bearing isolates as separate phenotypes. It is notable that one tumor-bearing isolate, CRC2652, maintained rapid growth throughout the 7-h observation period. It is possible that there is allowable diversity in proliferation rates in the tumor microenvironment as a means of maximizing survival through rapid generation of advantageous mutants. We hypothesize that once the strain is able to penetrate into the tumor, the tumor selects for cells in which the flagellar biosynthesis can be suppressed to reduce unnecessary energy consumption. More importantly, the population may be abandoning flagella as a means to evade the immune system, as flagella are known to pose a liability for bacteria during infection (21). We do not anticipate this to be of concern, as the strain itself remains attenuated due to the auxotrophic mutations, though future studies could explore the toxicity of recovered strains in 50% lethal dose (LD_{50}) studies; additional attenuating mutations may be necessary for the development of clinical strains. While other studies have demonstrated enhanced tumor colonization by the use of flagella (22), our direct observations indicated repression of the flagellar phenotype 3 days after tumor colonization. This indicates that genetic manipulation of the flagellar mechanisms may be an advisable technique to enhance therapeutic value. We acknowledge that differences in incubation times between passage through tumor-bearing (3 days) and TRAMP-negative (21 h) mice could be a confounding variable in the analysis of flagellar presence in the population, but the non-tumor-bearing mice cleared the strain from the body too rapidly, presenting an unavoidable experimental constraint. Since *S.* Typhimurium colonization and proliferation in the tumor environment occur rapidly (within 15 h) under *in vivo* conditions (23), we believe that the major selective forces establishing the tumor microenvironment as different from the host environment occur within the first 20 h of incubation, thus minimizing the impact of dissimilarities between incubation times on observed differences in bacterial phenotypes. In future studies, we will measure the number of *S.* Typhimurium generations that pass during this difference in time to quantify and characterize the additional selec-

tive pressure that could be arising due to this experimental constraint. We propose that these observed phenotypic differences are a result of an environmental selection for particular phenotypes and not randomly arising mutations of the injected bacterial population. Recovery of a majority of *S.* Typhimurium isolates with lower growth rates argues for selection in the tumor, since slow growth is a phenotype that one would normally expect to be found in a minority of the bacterial population.

The Biolog substrate plate assays revealed variability in substrate utilization among all of the *S.* Typhimurium strains examined. In particular, some tumor isolates lacked sucrose and adonitol metabolism. We attribute this variability and the loss of some substrate utilization by tumor isolates to selection pressures of the changing tumor environment during *Salmonella*-induced necrosis. Strains may be deriving nutrients from dying tumor cells, altering the metabolic profile of the *Salmonella* population. The high rate of glycolysis and its subsequent by-products in tumors (or products of the Warburg effect, where cancer cells depend heavily on glycolysis for energy production [24]) creates an available nutrient profile that is distinct from those of other environments of *S.* Typhimurium, which could be applying pressures that maintain the glycolic acid and glyoxylic acid metabolism in tumor isolates (25). Additionally, it has been shown that particular DNA motifs and promoters for metabolic utilization are preferentially upregulated in tumors (26), which may be affecting the changed metabolic profile of the population that inhabits the prostate tumor. These results emphasize the impact that physiological conditions have on eventual strain metabolism, as illustrated in the differences in substrate utilization in Table 4. Tumors also exhibit a gradient of metabolites (27), which would favor metabolic diversity within a population invading a tumor. Variability may be permissible or even favorable in terms of metabolic utilization of substrates for survival, whereas in the healthy host, deviants from allowable metabolic utilization are selected out of the system. Overall, we posit that this variability, along with the trends such as bacteriophage resistance maintenance, loss of flagella, lower growth rates, and changed substrate metabolism, illustrates various strategies adopted by the *Salmonella* population—under the selective pressure of the prostate tumor—to optimize the balance between minimizing energy consumption and maximizing survival and proliferation within the tumor.

Recommendations for further studies. We present these findings as an initial assessment of phenotypic changes that can occur to a therapeutic bacterial population during passage through a tumor and also acknowledge that the relatively small samples of isolates studied may not represent the breadth of evolutionary changes occurring within the population. Thus, to boost the statistical rigor of the claims made in this study, our next line of investigation is repeating these assays with a wider range of strain isolates from several TRAMP mice, in addition to the studies that we are pursuing as indicated in our discussion. Additionally, because the therapeutic use dictated that we inject the mice with a relatively small number of bacteria, which likely had little genotypic diversity, it is important to assess the number of cells to which the original population grew. In addition to assessing generation time as previously discussed, we also propose injecting $\sim 10^5$ cells into a tumor sample of known weight, incubating the sample overnight, and performing viable plate counts in the *in vitro* sample as a way of measuring the potential bacterial growth that a given amount of tissue could support and understanding how spontaneous mutations to create a diverse population could undergo natural selection. Initial explorations of this nature have already occurred, as described in this observation. To best assess the selection pressures of the tumor environment and how they may impact patient safety when tumors are treated with clinical *Salmonella*, we suggest a study in which all cells from the mouse tumor are collected and *Salmonella* strains are sorted via fluorescence-activated cell sorting (FACS), from which strains can be placed in the Biolog plates for a more exhaustive metabolic assay. This will include the extraction and analysis of *Salmonella* from non-tumor-bearing liver, spleen, and prostate tissue, if present. Alternatively, the sorted cells may also be directly sequenced or used to extract mRNA to create a *Salmonella* population transcriptome profile to compare metabolic data. Characterizing changes in the *Salmonella* population genome, transcriptome, or proteomes would be of particular importance because we have examined only the phenotypically different colonies of *Salmonella* and therefore it is likely that we have missed relevant phenotypes that do not produce different colony morphologies in rich medium. We find these assays to be valuable in their potential to monitor the rate and stages of metabolic change in the bacterial population within the tumor environment over time, give insight into the evolution of the bacterial population as its environment changes due to *Salmonella*-induced necrosis of tumor cells (and subsequent environmental pressures), and possibly shed light on the mechanisms of tumor destabilization taking place during tumor inoculation and persistence (for example, by consuming or altering molecules that inhibit the immune system in localized tumor regions). A longer-term tracking of tumor-associated strains (longer than 3 days) could reveal properties of long-term selective pressures. Injecting mice with *Salmonella*, sacrificing them on each progressive day, and examining harvested samples would provide insight into the average length of *Salmonella* persistence in the tumor-bearing mouse and the phenotypic traits that the tumor microenvironment selects for on a long-term scale. Finally, to address safety concerns more deliberately, harvested isolates may be injected back into TRAMP mice and other cancer models for toxicity studies, and efficacy may be further addressed via invasion assays by coincubation of isolates from tumor-bearing mice and healthy mice in *in vitro* prostate cancer cell lines.

MATERIALS AND METHODS

Bacterial strains and culturing methods. *S.* Typhimurium strains derived from wild-type LT2 were used in this study. Table 1 shows complete strain information. All *S.* Typhimurium strains were grown on nutrient Luria-Bertani (LB) agar plates (25 g/liter LB powder, 15 g/liter agar [Fisher BioReagents] in deionized water) supplemented with 200 μg/ml thymine (Acros Organics) at 37°C overnight. Plates for ampicillin-resistant strains included an additional 50 μg/ml ampicillin (Sigma). Strains were cultured in liquid medium by stab-inoculating 3 ml LB broth (25 g/liter LB powder in deionized water supplanted with 200 μg/ml thymine and an additional 50 μg/ml ampicillin, if required) with single colonies and incubating them in a 37°C water bath shaker. For antibiotic resistance studies, strains were cultured on LB-thymine plates and liquid medium was supplemented with 50 μg/ml ampicillin, as applicable.

Injection procedures and harvesting/recovery of *S.* Typhimurium. Male C57B16-Tg (tumor necrosis factor [TNF]-receptor-related apoptosis-mediated protein) 8247Ng/J (TRAMP) mice (Jackson Laboratory; as characterized in reference 28) aged 6 to 12 months were used to collect tumor-passaged and non-tumor-passaged *Salmonella*. All University of Missouri (Columbia, MO) institutional guidelines for animal care and use were followed. Overnight cultures of CRC2636 were grown in

100 ml of liquid medium. The culture was centrifuged and resuspended in 1× phosphate-buffered saline (PBS) (Fisher BioReagents), from which 1 ml was then serially diluted into PBS to a concentration of 10^6 CFU/ml. Tumor-bearing and non-tumor-bearing mice were injected intraperitoneally with 0.1 ml of 10^6 CFU/ml CRC2636, and the remaining solution was used for viable plate counts on nutrient plates to determine how many bacteria were injected. Genitourinary prostate tumor samples from tumor-bearing mice were collected 3 days after injection; spleen and liver samples from non-tumor-bearing mice were collected 21 h after injection. Samples were homogenized in PBS, serially diluted, and then plated on nutrient plates and incubated at 37°C to isolate individual colonies for further characterization. Two tumor-bearing mice and two healthy control mice were used to collect samples; all bacterial samples from tumors described in this paper came from a single tumor isolate.

Bacteriophage susceptibility assays. Sensitivity to phage was assessed by adding 5 μl of each phage to Evans blue uranine (EBU) green acidity differential plates (5 g/liter yeast extract [Fisher], 10 g/liter tryptone [BD], 2.5 g/liter glucose [Fisher], and 15 g/liter agar in deionized water, with the addition of 1.25 ml/liter 1% Evans blue [Aldrich], 2.5 ml/liter 1% uranine [Sigma], and 20 ml/liter 25% K_2HPO_4 [Fisher] after autoclaving) covered in a mixture of 3 ml top agar (6.5 g/liter agar, 5 g/liter tryptone-peptone [Difco], 24.4 g/liter LB powder in deionized water) and 200 μl of the *S.* Typhimurium strain being assessed. Lysis patterns on plates were examined after a 24-h incubation period at 30°C and are indicated in Table 2. The six phages used in this study were chosen after preliminary supernatant streak testing revealed the most consistently lysing phage out of an initial set of 20 different phages. ES-18, known to consistently lyse *S.* Typhimurium, was used as a control phage in order to ensure that all recovered strains were indeed *S.* Typhimurium. All phage were grown on *S.* Typhimurium strain CRC2754 (Table 1 gives strain information) with broth cultures centrifuged at 4,200 rpm for 17 min. The supernatant was poured into glass vials to which chloroform was added. Assays were performed in triplicate.

Motility assays. Petri dishes containing semisolid motility agar (25% top agar [6.5 g/liter agar, 5 g/liter tryptone-peptone, 24.4 g/liter LB powder in deionized water], 75% soft agar containing 2 g/liter agar, 7.5 g/liter tryptone-peptone in deionized water) were stab-inoculated in the center with liquid cultures of each strain, placed in boxes containing water as a moisture source, and incubated at 37°C. After 24 h, new motility plates were stab-inoculated in the center with cultures taken from the outer periphery of the motility ring from the previous day. Assays were performed in triplicate; the average diameter of strain growth was noted at various time points as indicated in Table 3.

Growth rates. Comparative growth rates of tumor-bearing *S.* Typhimurium isolates and non-tumor-bearing isolates were assessed by measuring the optical density (OD) of each strain at hourly intervals. Single colonies were picked from thymine-supplemented LB plates and cultured in 3 ml LB-thymine broth in a 37°C shaker overnight. All samples were diluted to an OD of 0.05 in 10 ml thymine-supplemented LB broth and placed in a 37°C shaker; 1-ml samples were measured in a spectrophotometer every hour for 7 h. Viable plate count dilutions were performed at 0, 1, 3, 5, and 7 h to obtain CFU/ml data on thymine-supplemented LB plate medium. Growth rate determination is the result of at least two independent assays.

Transmission electron microscopy. Isolate morphology was examined by centrifuging 3 ml of overnight cultures at 4,000 rpm for 5 min and resuspending them in 3 ml deionized water twice. For microscopy, 4 μl of the resuspension was applied to carbon-coated films on copper grids and negatively stained using 4% uranyl acetate. Specimens were viewed using a JEOL-1400 transmission electron microscope located in the University of Missouri—Columbia's Electron Microscopy Core Facility. Microscopy images are shown in Fig. 2.

Metabolic utilization assays. Normal saline was inoculated with 24-h cultures grown on Trypticase soy agar plates (BD) (30 g/liter Trypticase soy broth, 15 g/liter agar, where soy broth components included 17 g/liter pancreatic digest of casein, 3 g/liter papaic digest of soybean meal, 5 g/liter NaCl, 2.5 g/liter K_2PO_4, and 2.5 g/liter dextrose) from which 150 μl of the inoculated saline at 53% to 59% turbidity was transferred into each of the 96 wells (each containing a different carbon substrate) of an ES MicroPlate plate (Biolog; now available as PM-1 MicroPlate), and substrate utilization was observed after 24 h at 37°C.

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