Supporting Information

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SI Materials and Methods

Plasmids. Three plasmids were generated: pQS-GFP, pLacGFP, and pQS-SAH (Fig. S3; GenBank accession numbers KP294373, KP294374, and KP294375). pQS-GFP and pLacGFP controlled expression of GFP (GFPmut3) with quorum sensing (Fig. 1*A*) or the constitutive *p(Lac)* promoter as a control, respectively. pQS-SAH controlled expression of the bacterial toxin *Staphylococcus aureus* α-hemolysin (SAH) with quorum sensing (Fig. S3*A*). All plasmids contained aspartate–semialdehyde dehydrogenase (*asd*) to create a balanced lethal system that would retain plasmids in mice without antibiotics. Genes were cloned from plasmids pACluxInv, pluxI-Gfpmut3-luxR, placGFPmut3, pYA3332, and the *S. aureus* strain MW2, which were kind gifts from Dr. Christopher Voigt (University of California, San Francisco, CA), Dr. Lianhong Sun (University of Massachusetts, Amherst, MA), Dr. Voyich-Kane (Montana State University, Bozeman, MT), and Vion Pharmaceuticals (New Haven, CT). Plasmid cloning was performed in *Escherichia coli* DH5α (Invitrogen) and transformed into VNP200010.

To create pQS-GFP, *luxI* and *asd* were amplified from pACluxInv and pYA3332 and subcloned into pluxI-Gfpmut3-luxR, which contains *luxR* and *gfpmut3* controlled by *p*(*luxI*). Amplification of *luxI* used forward primer 5′-TTCGGCCGGAA-TAAACGCAAG-3′ (*EagI* site underlined) and reverse primer 5′-GCACATGTATCTTGAATCATTCCAT-3′ (*PciI* site underlined). Amplification of *asd* used forward primer 5′-GCTCAT-GACGTACGTTTTCGTTCCATTG-3′ (*BspHI* site underlined) and reverse primer 5′-ATACTAGTATCTGCGTTTACTCTT-GTATTACG-3′ (*SpeI* site underlined). To create pLacGFP, *asd* was amplified from pYA322 and subcloned into placGFPmut3. Amplification of *asd* used forward primer 5′-GCGAATTCG-TACGTTTTCGTTCCATTG-3′ (*EcoRI* site underlined) and reverse primer 5′-ATGACGTCATCTGCGTTTACTCTTGTA-TTACG-3′ (*AatII* site underlined). To create pQS-SAH, the gene encoding SAH was amplified from the genome of *S. aureus*, strain MW2 and subcloned into pQS-GFP, replacing *gfpmut3*. Amplification of *sah* used forward primer 5′-TAGCTAGCTC-GTTTAAAAAAATAGAAG-3′ (*NheI* site underlined) and the reverse primer 5′-ATCCTGCAGGTTAATTTGTCATTTCTT-CT-3′ (*SbfI* site underlined). All restriction enzymes were purchased from New England Biolabs.

Density-Dependent Expression by QS *Salmonella*. To measure density dependence, QS and control *Salmonella* were grown overnight in LB and diluted to 10^5 cfu/mL. Every hour, the optical density (OD) was measured at 600 nm and the fluorescence was measured at excitation and emission wavelengths of 465 and 505 nm. OD was calibrated by plating geometric dilutions of a culture with a known density and counting colonies. The linear conversion factor was 0.2 $OD_{600} = 1 \times 10^8$ cfu/mL. To test robustness of induction density, cultures of QS *Salmonella* were grown from single colonies. Two aliquots were diluted to 10^5 cfu/mL when the culture reached an OD of 0.1 (low dilution) and 1.0 (high dilution). Every hour, the OD and fluorescence of both cultures were measured.

Measurement of SAH Expression and Induced Cell Death in Vitro. Single colonies of QS-SAH *Salmonella* were grown overnight in LB and diluted to 10^5 cfu/mL. At ODs of 0.54 (low density, 2.7×10^8 cfu/mL) and 3.0 (high density, 15×10^8 cfu/mL), bacteria were pelleted by centrifugation and supernatants were extracted. Supernatant fractions were concentrated 100-fold by centrifugal ultrafiltration (Millipore). The supernatant buffer was exchanged three times with PBS and purified through a 0.22-μm filter. To compare protein expression on an individual bacterium basis, 2.5 μl high-density concentrate and 14 μl low-density concentrate were diluted into 15 μl PBS. These samples were diluted 1:1 with 15 μl Laemmli reducing buffer and immunoblotted using sheep anti-SAH polyclonal antibody #ab15948 (Abcam). MCF-7 human mammary carcinoma cells (American Tissue Type Collection) were seeded at a density of 30,000 cells per well in 48-well plates and grown to 70% confluence. Media were aspirated and replaced with DMEM, supplemented with 2 μM of ethidium homodimer (EtHd; Invitrogen). Concentrated supernatant from high- and low-density QS-SAH cultures was added at volumes proportional to the densities of the original bacterial cultures. After 16 h, transmitted and red fluorescent images were captured for each well. The total number of cells was counted based on transmitted images and cells were considered dead if they contained EtHd in the red fluorescent images.

Measurement of Protein Expression Within Tumor Tissue in Vitro. A microfluidic tumor-on-a-chip device containing LS174T colon carcinoma cells was used to measure bacterial protein expression in tissue. Cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% (vol/vol) FBS (Sigma-Aldrich) at 37 °C and 5% (vol/vol) CO_2. Multicellular tumor spheroids were formed by growing 2.5×10^4 cells per mL in flasks coated with poly(2-hydroxyethyl methacrylate) (Sigma-Aldrich) for 12 d. Devices were fabricated using soft lithography (1–3) and were sterilized by flushing with 70% ethanol, followed by PBS to remove air and residual ethanol.

Spheroids were inserted into 1,000 × 300 × 150-μm cuboidal chambers and were incubated for 24 h at 37 °C in an enclosed, humidified environment. Devices were supplied with Hepes-buffered (25 mM) DMEM flowing at 3.0 μl/min. QS and control *Salmonella* were grown overnight, centrifuged, and resuspended in DMEM with 10% FBS at a density of 10^5 cfu/mL. Bacteria-containing medium was administered to devices for 1 h at time 0 and then switched to bacteria-free medium. Images of chambers were acquired for 30 h starting 23 h after bacterial inoculation (Olympus). To capture entire chambers (1,000 × 300 μm), two images (867.15 × 660.68 μm) were obtained at 10× and tiled together in IPLab (BD Bioscience). Transmitted and fluorescence images were captured at 1-h intervals, using 470-nm excitation and 525-nm emission filters (Chroma). Area fractions of GFP expression were determined for each tissue as a function of time using ImageJ (NIH Research Services Branch), after subtracting background fluorescence.

Measurement of Cell Death Induced by QS-SAH in Tumor Tissue in Vitro. Microfluidic devices were used to measure induced cell death by QS-SAH in tissue. Spheroids were grown and inserted into microfluidic devices, which were supplied with Hepes-buffered DMEM. QS-GFP and QS-SAH *Salmonella* were grown overnight, centrifuged, and resuspended in DMEM with 10% FBS at a density of 10^6 cfu/mL. Bacteria-containing medium, supplemented with 2 μM EtHd, was administered to devices for 1 h at time 0 and then switched to bacteria-free medium, also containing 2 μM EtHd. Transmitted and fluorescence images were acquired every 30 min for 30 h. EtHd was identified using 546-nm excitation and 590-nm emission filters (Chroma). Average fluorescence intensity was measured for every tissue as a function of time using ImageJ. Background fluorescence was

subtracted and intensities were normalized by the values from control tissues at 8 h.

Tumor Formation and *Salmonella* Administration. Tumors were formed by s.c. injecting 50,000 4T1 mammary carcinoma cells (American Tissue Type Collection), suspended in PBS, into the flank of 8-wk-old BALB/c mice. Before implantation, 4T1 cells were grown in RPMI-1640 with 10% FBS. Caliper measurements were taken regularly to monitor tumor growth. When tumors reached 500 mm^3, 2×10^6 cfu midlog phase QS and control *Salmonella*, suspended in 100 μl PBS, were injected via the tail vein. Mice were killed when tumors reached 2,000 mm^3. Excised tumors and livers were cut in half. One half was fixed in 10% formalin and embedded in paraffin, and the other half was weighed, minced in saline, and plated. Colonies on LB-agar plates were counted after growth at 37 °C for 24 h to determine bacterial densities in tissues.

Immunofluorescence Labeling and Image Acquisition. *Salmonella* and GFP were identified by immunofluorescence. Equatorial sections, 5 μm thick, were cut from excised tumors and livers. Sections were fixed, embedded in paraffin, deparaffinized, and rehydrated. Antigens were retrieved by soaking sections in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 20 min. Proteins were blocked with DAKO serum-free blocking solution (Agilent Technologies) for 20 min. Sections were probed with a 1:200 dilution of rabbit anti-*Salmonella* (Abcam) and a 1:50 dilution of goat anti-GFP (Abcam) overnight, followed by incubation with a 1:200 dilution of Alexa Fluor 546-conjugated donkey anti-rabbit antibody and a 1:50 dilution of Alexa Fluor 488-conjugated donkey anti-goat antibody at room temperature for 1 h. Processed sections were counterstained with DAPI-containing mounting medium (Vecta Labs), mounted with coverslips, stored at 4 °C, and protected from light. Images of entire sections were obtained by tiling together 250–650 adjacent 867.15 × 660.68-μm frames acquired at 10× using epifluorescent microscopy. *Salmonella*, labeled with Alexa 546, were identified using 546- and 590-nm excitation and emission filters (Chroma). GFP, labeled with Alexa 488, was identified using 470- and 525-nm excitation and emission filters (Chroma).

Bacterial and GFP Distribution Analysis. *Salmonella* and GFP distribution was quantified using ImageJ. Red and green fluorescence images were thresholded and converted into binary images to identify regions (pixels) that contained *Salmonella* and GFP. Artifacts were manually eliminated based on morphology and fluorescence intensity. *Salmonella* colonies and their centers of mass were identified by particle analysis in ImageJ. The density of *Salmonella* surrounding each colony was determined by counting the number of red fluorescent pixels in a 150-pixel (197-μm) circle around the colony center. An individual red pixel was assumed to be one bacterium. The average radial distance of all neighboring bacteria was determined by counting the number of *Salmonella* within 5-pixel annuli of increasing radius from the colony center. Averages were weighted by the inverse of the area of each annulus. Colonies were considered to be induced if GFP pixel was within 25 pixels. Fractions of expressing colonies were determined for colony groups of increasing density and radius.

Mathematical Modeling of 3OC6HSL Transport and Production. A mathematical model was used to predict the concentration of 3OC6HSL in tumor tissue. The model balanced 3OC6HSL accumulation and diffusion (Eq. **1**). The concentration of 3OC6HSL was modeled as release from a point source in 3D spherical space. Molecules of 3OC6HSL were constantly released from the center of mass of source bacterial colonies at rate, m (moles/time), which switched from zero to maximum production (m_{max}) above a critical density (ρ_{crit}). Release rates were constant and dependent on the density of the source colony (Eq. **1**, right). The effective diffusion coefficient (D_{eff} in Eq. **1**, left) relates to the diffusion coefficient in aqueous medium (D_0) by the inverse square of the tortuosity around cellular barriers ($D_{eff} = D_0/\tau^2$; ref. 4). At early times, the 3OC6HSL concentration was zero throughout the tissue. Far from source colonies the concentration of 3OC6HSL was zero.

$$C_s|_{t=0} = 0 \quad C_s|_{r\to\infty} = 0. \qquad \textbf{[S1]}$$

The analytical solution of Eq. **1** is a complementary error function that is dependent on radial distance (r) and time (t).

$$C_s = \frac{m}{4\pi D_{eff} r}\operatorname{erfc}\left(\frac{r}{2(Dt)^{1/2}}\right). \qquad \textbf{[S2]}$$

At long times ($t\to\infty$), Eq. **S2** can be simplified to a function that is linearly dependent on the 3OC6HSL production rate, m, and inversely dependent on radius, r, and the effective diffusion coefficient, D_{eff}.

$$C_s = \frac{m}{4\pi D_{eff} r}. \qquad \textbf{[S3]}$$

Normalizing Eq. **S3** for concentration ($\overline{C}_s = C_s/C_q$) and radial distance ($\bar{r} = r/r_c$) results in Eq. **S4**, in which $\overline{C}_s$ is linearly dependent on dimensionless parameter Q and inversely dependent on $\bar{r}$.

$$\overline{C}_s = \frac{Q}{\bar{r}} \qquad Q = \frac{m}{4\pi D_{eff} C_q r_c}. \qquad \textbf{[S4]}$$

For the entire range of effective diffusion coefficients of biological molecules in tumor tissue (4), this concentration profile approaches steady state within 1 h (5). Because mice were killed at least 1 wk after bacterial injection, the 3OC6HSL concentration profile was assumed to be at steady state. This system reaches steady state because, at long times, the molecular fluxes through all spherical shells surrounding a source colony are equal, and equal to the production rate, m. When t is large, the complementary error function term in Eq. **S2** approaches a value of 1. This limit indicates that, at long times, 3OC6HSL is produced, but that production has an infinitesimally small effect on the concentration profile.

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3. Walsh CL, et al. (2009) A multipurpose microfluidic device designed to mimic microenvironment gradients and develop targeted cancer therapeutics. *Lab Chip* 9(4): 545–554.
4. Pluen A, et al. (2001) Role of tumor-host interactions in interstitial diffusion of macromolecules: Cranial vs. subcutaneous tumors. *Proc Natl Acad Sci USA* 98(8):4628–4633.
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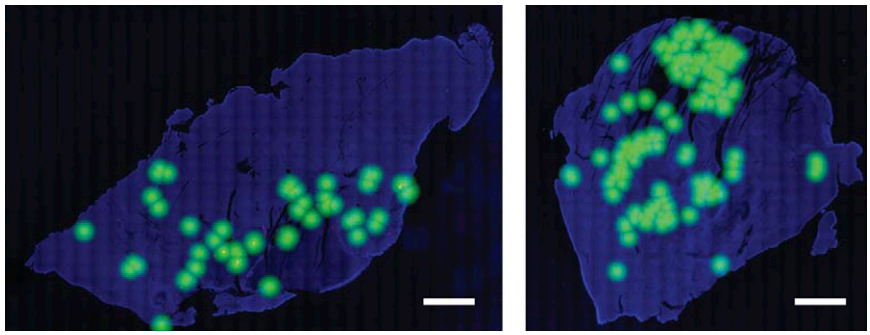


Fig. S1. Locations of GFP expression in two tumors containing QS *Salmonella*. Locations were enhanced by dilation of GFP pixels. (Scale bar, 5 mm.)

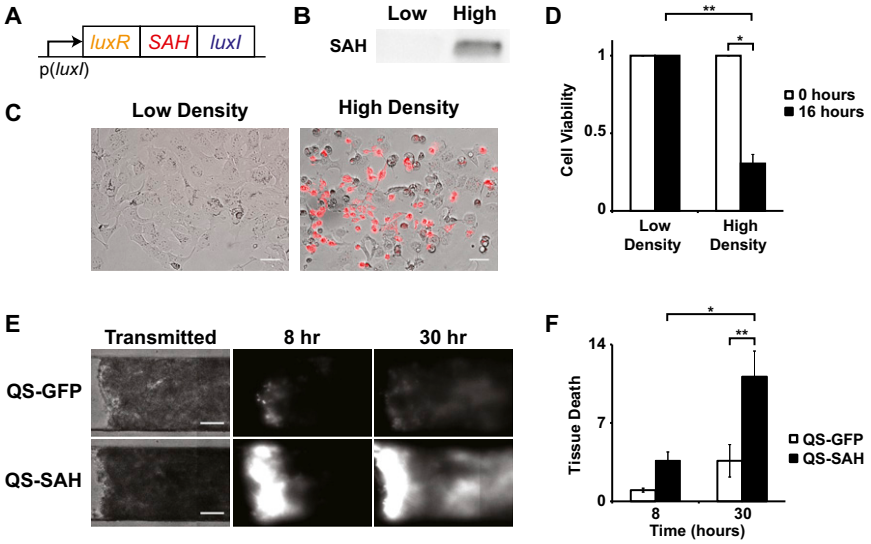
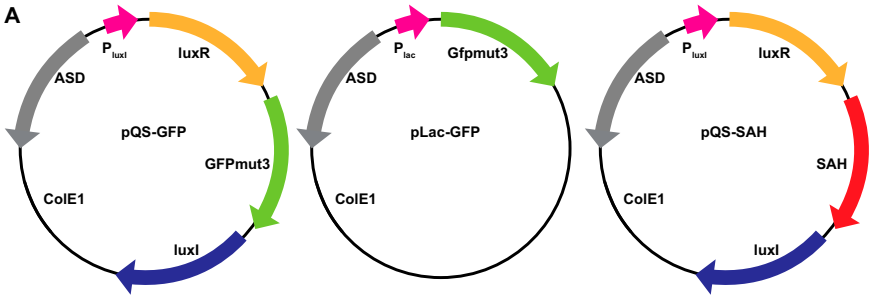


Fig. S2. QS-controlled SAH kills tumor cells. (*A*) The therapeutic molecule, SAH, was integrated into the QS system between *luxR* and *luxI*. (*B*) QS-SAH *Salmonella* were grown to two densities: low, 2.7×10^8 cfu/mL; and high, 15×10^8 cfu/mL. Supernatants were extracted and diluted in PBS, proportional to culture density. The high-density culture produced SAH, and none was detected in the low-density culture. The density of the low-density culture is 18,000-fold higher than the density measured in livers (1.5×10^4 cfu/g, Fig. 3*A*). (*C*) Supernatants from the low- and high-density QS-SAH cultures were added to plates of MCF-7 human mammary carcinoma cells. Cells were stained for viability with EtHd, red. At 16 h, no dead cells were observed in low-density plates and many were present in high-density plates. (*D*) Between 0 and 16 h, supernatant from high-density QS-SAH cultures significantly reduced cancer-cell viability (*$P < 0.05$, $n = 3$). At 16 h, viability in high-density plates was significantly lower than low-density plates (**$P < 0.05$; $n = 3$). No death (viability = 1.0) was observed in low-density plates at 0 or 16 h. (*E*) QS-SAH and control QS-GFP *Salmonella* were administered to tumor tissue in a microfluidic device at a density of 10^5 cfu/mL and were cleared after 1 h. Tissue was stained for viability with EtHd. Fluorescence intensity at 8 and 30 h indicates the extent of cell death. The distribution of bacteria in the device is dependent on penetration and proliferation, the same mechanisms that control distribution in tumors in vivo (1). (Scale bar, 100 μm.) (*F*) Average fluorescence from EtHd-stained tissues (*E*), normalized by the intensity of control tissues at 8 h. From 8 to 30 h, QS-SAH *Salmonella* significantly induced cell death (*$P < 0.05$; $n = 4$). QS-SAH killed significantly more tissue than control *Salmonella* (**$P < 0.05$; $n = 4$).

1. Zhang M, Forbes NS (2015) Trg-deficient Salmonella colonize quiescent tumor regions by exclusively penetrating or proliferating. *J Control Release* 199:180–189.

A



B

Gene/Promoter	Accession Number
PluxI	HQ436485
luxI	AF170104.1:1187..1768
luxR	complement(AF170104.1:216..968)
Gfpmut3	AIO09750
SAH	complement(BA000033.2:1140262..1141221)
ASD	AF015781
Plac	complement(U00096:366350..366379)

Fig. S3. Plasmid maps and gene accession numbers. (*A*) QS-GFP was the primary construct, used throughout to test induction at high density in culture and in colonies in tumor tissue. The pLac-GFP construct constitutively expresses GFP and served as a control throughout. QS-SAH was used to test induction of a toxic molecule (SAH) by the QS architecture. (*B*) Accession numbers of genes and promoters used to construct the three plasmids in *A*.