Acoustic reporter genes for noninvasive imaging of microorganisms in mammalian hosts

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The mammalian microbiome has many important roles in health and disease1–3, and genetic engineering is enabling the development of microbial therapeutics and diagnostics4–7. A key determinant of the activity of both natural and engineered microorganisms in vivo is their location within the host organism8,9. However, existing methods for imaging cellular location and function, primarily based on optical reporter genes, have limited deep tissue performance owing to light scattering or require radioactive tracers10–12. Here we introduce acoustic reporter genes, which are genetic constructs that allow bacterial gene expression to be visualized in vivo using ultrasound, a widely available inexpensive technique with deep tissue penetration and high spatial resolution13–15. These constructs are based on gas vesicles, a unique class of gas-filled protein nanostructures that are expressed primarily in water-dwelling photosynthetic organisms as a means to regulate buoyancy16,17. Heterologous expression of engineered gene clusters encoding gas vesicles allows *Escherichia coli* and *Salmonella typhimurium* to be imaged noninvasively at volumetric densities below 0.01% with a resolution of less than 100 μm. The ability of engineered cells in vivo to be imaged in proof-of-concept models of gastrointestinal and tumour localization, and develop acoustically distinct reporters that enable multiplexed imaging of cellular populations. This technology equips microbial cells with a means to be visualized deep inside mammalian hosts, facilitating the study of the mammalian microbiome and the development of diagnostic and therapeutic cellular agents.

Gas vesicles comprise all-protein shells with sizes of approximately 200 nm that enclose hollow interiors, and allow dissolved gases to permeate freely in and out while excluding water18. We recently discovered the ability of these proteins to scatter sound waves and thereby produce ultrasound contrast19. However, the ability of the multi-gene clusters encoding gas vesicles to serve as reporter genes in heterologous species has not been demonstrated. Gas vesicles are encoded in their native bacterial or archaean hosts by operons of 8–14 genes, which include the primary structural protein GvpA, the optional external scaffolding protein GvpC, and secondary proteins that function as essential minor constituents or chaperones17. As a starting point for developing acoustic reporter genes (ARGs), we chose a compact *E. coli*-compatible gas vesicle gene cluster from *Bacillus megaterium* (Fig. 1a; top left). Although cells containing this construct were able to produce small, bicone-shaped gas vesicles (Fig. 1b, c; left), its expression did not result in bacteria that were detectable by ultrasound (Fig. 1d; left), most probably because the small gas vesicles produced from this construct have weak acoustic scattering. At the same time, transforming *E. coli* with a gas vesicle gene cluster derived from the cyanobacterium *Anabaena flos-aquae*, the gas vesicles of which are highly echogenic18,20, did not result in gas vesicle expression. Given the high sequence homology of GvpA between organisms (Extended Data Fig. 1), we hypothesized that a combination of the structural gvpA genes from *A. flos-aquae* with the accessory genes gvpR–gvpU from *B. megaterium* (Fig. 1a; middle) would result in the formation of gas vesicles with characteristics favourable for ultrasound imaging. Indeed, expression of this engineered gene cluster resulted in *E. coli* containing gas vesicles with substantially larger dimensions compared to the *B. megaterium* operon, and these nanostructures appeared to occupy a greater fraction of intracellular volume (Fig. 1b, c; middle). Notably, these cells produced robust ultrasound contrast compared to green fluorescent protein (GFP) controls (Fig. 1d; middle). Further engineering comprising the addition of a gene encoding the *A. flos-aquae* scaffolding protein GvpC (Fig. 1a; right) resulted in wider and more elongated gas vesicles that more closely resembled those native to *A. flos-aquae* (Fig. 1b, c; right), and generated stronger ultrasound contrast (Fig. 1d; right). We refer to this optimized genetically engineered construct as acoustic reporter gene 1 or arg1.

To confirm that the ultrasound signal from arg1-expressing cells is due to the presence of gas vesicles, we applied acoustic pulses with amplitudes above the critical collapse pressure of the gas vesicles20. In purified samples, this resulted in the immediate collapse of these protein nanostructures and dissolution of their gas contents, eliminating ultrasound contrast18,20. As expected, the application of high-pressure pulses made cells expressing arg1 invisible to ultrasound (Fig. 1d). The ability of ARG-based contrast to be erased in situ is used throughout this study to confirm the source of acoustic signals and subtract background. arg1 expression resulted in gas vesicle contents of 9.4 ± 0.4 mg g⁻¹ *E. coli* (*n* = 3, mean ± s.e.m.), corresponding to approximately 100 gas vesicles per cell. These nanostructures occupy roughly 10% of the intracellular space. Acoustically silent cells expressing the *B. megaterium* gene cluster produced a similar quantity of gas vesicle proteins (9.7 ± 1.5 mg g⁻¹, *n* = 3), underscoring the importance of genetic engineering in producing intracellular nanostructures with the appropriate size and shape to be detected by ultrasound. A fraction of arg1-expressing cells was buoyant in aqueous medium (Extended Data Fig. 2a, b), suggesting that gas vesicles occupy more than 10% of their volume. However, the expected buoyant force on these cells, even at much higher expression levels, is weak compared to other forces such as flagellar thrust (Supplementary Table 1).

To determine the detection limit of ARG-expressing cells, we imaged a concentration series of *E. coli* transformed with arg1 (Fig. 2a). Cells at concentrations as low as 5 × 10⁶ cells ml⁻¹ produced a detectable signal (Fig. 2a, b). This equates to a roughly 0.005% volume fraction, or approximately 100 cells per voxel based on cubic voxel dimensions of 100 μm. This sensitivity should be sufficient for many *in vivo* scenarios21. Furthermore, bacteria enriched for buoyancy before imaging provide a 2.4-fold higher signal (Extended Data Fig. 2c, d), suggesting that sensitivity could be improved further by optimizing ARG expression.

To test whether ARGs could provide a read-out of state-dependent genetic pathways, we placed arg1 under the control of a promoter.
regulated by the chemical inducer isopropyl-α-D-thiogalactoside (IPTG). Ultrasound signals from \textit{E. coli} expressing ARGs in this configuration followed the expected dose–response curve of IPTG-controlled expression (Fig. 2c, d), confirming their ability to serve as the output signal for engineered genetic circuits. Significant ultrasound contrast could be observed 4 h after IPTG induction (P = 0.01, n = 4), and continued to increase during the 22-h culturing period (Extended Data Fig. 3).

To determine whether the expression of ARGs has any deleterious effect on host cells, we measured the growth curves of \textit{E. coli} expressing \textit{arg1} or \textit{arg2}. After induction, cells expressing both constructs continued to divide and reached similar saturation densities (Extended Data Fig. 4a). For both \textit{arg1} and \textit{GFP}, the final density was lower than in uninduced controls, as expected from the metabolic demand of protein expression\textsuperscript{22}. We also assessed the viability of ARG-expressing cells after ultrasound imaging and acoustic collapse. Transmission electron microscopy (TEM) images of cells acquired before and after exposure to collapsing acoustic pulses show that gas vesicles can be eliminated without any obvious cellular damage (Extended Data Fig. 4b). To examine the effect of ultrasound exposure on cell growth, we cultured \textit{E. coli} expressing \textit{arg1} as colonies on solid medium and applied acoustic collapse pulses to half of the agar plate. The collapse of gas vesicles in insonated cells was confirmed by a decrease in optical scattering (Extended Data Fig. 4c, d). After incubation for an additional 20 h, no significant difference was observed in the diameter of the insonated colonies compared to un-sonated controls, indicating that ultrasound exposure does not affect cell viability (Extended Data Fig. 4e). Notably, insonated colonies re-expressed gas vesicles during this period, as indicated by the restoration of pressure-sensitive light scattering (Extended Data Fig. 4f, f).

It is often informative to image more than one population of cells simultaneously, as done optically using spectrally distinct fluorescent proteins. Analogous acoustic multiplexing can be performed using genetic variants of gas vesicles that collapse at different pressures\textsuperscript{20} (Supplementary Note 1). To explore whether this could be done with ARGs, we constructed a new version of the ARG-expressing gene cluster containing a modified version of \textit{A. flos-aquae} gvp\textit{C}. Deletion or truncation of this outer scaffolding gene clusters. The region highlighted in grey was varied. Panels b–d are organized in columns that correspond to each of the variant constructs. b, TEM images of representative \textit{E. coli} cells expressing each construct. c, TEM images of gas vesicles isolated from \textit{E. coli} expressing each construct. d, Ultrasound images of agarose phantoms containing \textit{E. coli} expressing each construct or GFP. The cell concentration is 10\textsuperscript{8} cells ml\textsuperscript{–1}. Images in the bottom panels were acquired after acoustic collapse. Dotted blue outlines indicate the location of each specimen. Colour bar represents linear signal intensity. Scale bars, 500 nm (b), 250 nm (c) and 2 mm (d). All imaging experiments were repeated three times with similar results.

**Figure 1 | Genetic engineering of acoustic reporter genes.** a, Organization of acoustic reporter gene clusters; the region highlighted in grey was varied. Panels b–d are organized in columns that correspond to each of the variant constructs. b, TEM images of representative \textit{E. coli} cells expressing each construct. c, TEM images of gas vesicles isolated from \textit{E. coli} expressing each construct. d, Ultrasound images of agarose phantoms containing \textit{E. coli} expressing each construct or GFP. The cell concentration is 10\textsuperscript{8} cells ml\textsuperscript{–1}. Images in the bottom panels were acquired after acoustic collapse. Dotted blue outlines indicate the location of each specimen. Colour bar represents linear signal intensity. Scale bars, 500 nm (b), 250 nm (c) and 2 mm (d). All imaging experiments were repeated three times with similar results.

**Figure 2 | Imaging dilute bacterial populations and dynamically regulated gene expression.** a, Ultrasound images of \textit{arg1}–expressing \textit{E. coli} at various cellular concentrations, before and after acoustic collapse. b, Mean ultrasound contrast from \textit{E. coli} expressing \textit{arg1} and \textit{GFP} at various cell densities. Data are from three biological replicates, lines indicate the mean. AU, arbitrary units. c, Ultrasound images of \textit{E. coli} expressing \textit{arg1} after induction with various concentrations of IPTG. Cell concentration was 5 × 10\textsuperscript{8} cells ml\textsuperscript{–1}. d, Normalized ultrasound contrast as a function of IPTG concentration. Data are from three biological replicates, line shows a fit of the data with the Hill equation to facilitate visualization. Each imaging experiment was repeated three times with similar results. Scale bars, 2 mm.

**Figure 3 | Multiplexed imaging of genetically engineered reporter variants.** a, Diagram of the gvp\textit{A} and gvp\textit{C} sequences included in the \textit{arg1} and \textit{arg2} gene clusters. b, Ultrasound images of a gel phantom containing \textit{E. coli} expressing \textit{GFP} or \textit{arg2} (10\textsuperscript{8} cells ml\textsuperscript{–1}). Dotted blue outlines indicate the location of each specimen. c, TEM images of isolated \textit{arg2} gas vesicles. d, Ultrasound images of gel phantoms containing \textit{arg1} or \textit{arg2} before collapse, after collapse at 2.7 MPa and after collapse at 4.7 MPa (10\textsuperscript{8} cells ml\textsuperscript{–1}). e, Overlay of the blue and orange maps from spectral unmixing of \textit{arg2} and \textit{arg1}, based on the series of images in d. Scale bars, 2 mm (b, d, e) and 250 nm (c). Each imaging experiment was repeated three times with similar results.
The result with bioluminescent imaging, we introduced ECN expressing bacteria within the gastrointestinal tract, and to compare bioluminescent imaging, to visualize gene expression in microbial populations.

ECN (Enterococcus niger) is a probiotic microorganism capable of colonizing the mammalian gastrointestinal tract for 100 years to treat enteric infection and inflammatory bowel conditions. ECN has been used clinically in vivo to treat enteric infections and inflammatory bowel disease. ECN expressing arg1 proximal to the colon wall, and ECN expressing lux at the centre of the lumen.

To assess the ability of each modality to resolve the spatial distribution of bacteria within the colon, we injected the arg1 and lux cells into the centre or periphery of the colonic lumen. Ultrasound images clearly revealed the localization of ARG-expressing ECN cells in the appropriate region of the colon at concentrations of 10^9 cells ml^{-1}, which is within the range of certain commensal and therapeutic scenarios, and below the density reached by ECN in gnotobiotic models. Contrast ultrasound images were used to show the location of the bacteria within the context of other internal organs. Alternatively, ARG-expressing cells can also be seen in the colon in vivo ultrasound images. Contrast ultrasound images were used to show the location of the bacteria within the context of other internal organs.

The gut microbiome is a complex network of microorganisms that has been implicated in various diseases, including inflammatory bowel disease, obesity, and metabolic disorders. Understanding the distribution and function of these microorganisms is crucial for developing effective treatments.

Figure 4: Ultrasound imaging of bacteria in the gastrointestinal tract.

a. Diagram of gastrointestinal (GI) imaging experiment.

b. Representative TEM images of whole ECN cells expressing arg1 or the lux operon. Images were acquired from three biologically independent samples for arg1 and one for lux (approximately 35 cells imaged in each sample) with similar results.

c. Ultrasound images of a gel phantom containing ECN expressing arg1 or the lux operon. Experiment repeated five times with similar results.

d. Mean collapse-sensitive ultrasound signal in phantoms containing ECN cells expressing arg1 or lux. Line represents mean. (P = 0.0007 using a two-sided heteroscedastic t-test, n = 5).

After establishing the core capabilities of ARGs in vitro, we set out to demonstrate their detectability in vivo by imaging ARG-expressing cells in biologically relevant anatomical contexts. An important target for in vivo microbial imaging is the mammalian gastrointestinal tract, as seen in the effect of the gut microbiome on host health and the development of gastrointestinal-targeted microbial therapeutics.

Owing to its location deep inside the body, the gastrointestinal tract is difficult to image using optical techniques. To establish a proof of concept for ultrasonic imaging of microorganisms in this context, we expressed ARGs in a probiotic bacterial strain and assessed the ability of ultrasound to localize this bacterium inside the colon (Fig. 4a) in comparison with bioluminescent imaging.

The E. coli strain Nissle 1917 (ECN) is a probiotic microorganism capable of colonizing the mammalian gastrointestinal tract. ECN has been used clinically in humans for 100 years to treat enteric infection and inflammatory bowel conditions, and is a common chassis for therapeutic synthetic biology. ECN cells transformed with a plasmid expressing arg1 produced abundant gas vesicles (Fig. 4b) and ultrasound contrast (Fig. 4c, d). For comparison, we transformed ECN cells with the lux operon operon, which has previously been used to visualize gene expression in microbial populations in vivo using bioluminescent imaging. lux-expressing ECN cells produced no ultrasound contrast (Fig. 4c, d).

To establish a proof of concept for ultrasound imaging of ARG-expressing bacteria within the gastrointestinal tract, and to compare the result with bioluminescent imaging, we introduced ECN cells expressing arg1 or lux into the colons of anesthetized mice.
Finally, to facilitate future genetic engineering of ARGs, we assessed the amenability of these constructs to high-throughput screening. In fluorescent protein engineering, directed evolution has served as an effective approach to identify variants with new spectral and biochemical properties 31,32, often using mutant bacterial colonies as a convenient platform for high-throughput screening 32. To determine whether a similar approach could be used with ARGs, we developed a method to scan bacterial colonies with ultrasound (Fig. 5a). In this method, colonies are immobilized on agar plates with an over-layer of agarose, then scanned with an ultrasound transducer translated by a computer-controlled robot. This results in a series of transverse images that can be reconstructed to form an in-plane image of the plate (Fig. 5b). We used this technique to image a mixed plate of E. coli transformed with arg1, arg2 or GFP. Serial acoustic colony imaging (Fig. 5b) revealed three distinct colony populations (Fig. 5c and Extended Data Fig. 10), allowing the genotypes to be distinguished from each other with 100% accuracy (Fig. 5d). This result suggests that colony screening can discriminate acoustic phenotypes with sufficient accuracy to serve as a high-throughput assay for acoustic protein engineering.

Our study establishes engineered gas vesicle gene clusters as reporter genes for ultrasound, giving this widely used noninvasive imaging modality the ability to visualize genetically modified bacteria inside living animals. Future work will build on the in vitro and in vivo proofs of concept presented in this study to answer scientific and translational questions. This research will benefit from the development of ultrasound techniques to detect ARG signals and distinguish them from background (Supplemental Note 4), further genetic engineering to optimize the stability and host burden of ARG constructs, and expression of these reporters in a broader range of microbial species (Supplemental Note 5). In addition, it is ultimately desirable to express ARGs in mammalian cells.

We anticipate that the ARGs presented in this work are a starting point for future engineering of ultrasound reporter genes. Since their initial discovery as optical reporters, fluorescent proteins have been engineered, evolved and used in thousands of unforeseen optical imaging applications. Our findings that genetic engineering can be used to generate ARGs with distinct acoustic properties and that ARGs are amenable to colony-based high-throughput screening suggest that a similar trajectory may be available for this new technology.

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Letter reSeArCH

1.2 MPa hydrostatic pressure to eliminate any contribution to light scattering following induction, which had an optical density insufficient for loading, were Cells at 2% (w/v) agarose in PBS and casting wells using a custom 3D-printed template. Optics) measured the optical density of the sample at 500 nm. OD500nm was then imaged on a FEI Tecnai T12 transmission electron microscope equipped with a Gatan Ultrascan CCD. Images were processed with Fiji35.

Gas vesicle purification and quantification. Cells were centrifuged at 350g for 10 min. The supernatant containing the gas vesicles was transferred to 2 ml tubes and centrifuged for 2 h at 4 °C. The subnatant was removed with a 21.5-gauge needle, and the supernatant containing the gas vesicles was transferred to a clean tube. PBS was added to the gas vesicles in a threefold volume excess and centrifugation, removal of subnatant and PBS dilution was repeated twice. For ARG variants that do not produce a buoyant band of cells, the middle layer between the buoyant cells and the sedimented cells was removed and discarded. For ARG variants that do not produce a buoyant band, the supernatant was discarded. The remaining cells were resuspended in 8 ml SolutLyse-Tris (L200050 Genlanits) per 100 ml culture and 250 μl/Ml J-100s in a 2 h, 4 °C rotation. Subsequently, 10 μl-Ml β-DNase was added to the lysate and incubated for 10 min at 25 °C. The lysate was transferred to 2 ml tubes and centrifuged for 2 h at 4 °C. The subnatant was removed with a 21.5-gauge needle, and the supernatant containing the gas vesicles was transferred to a clean tube. PBS was added to the gas vesicles in a threefold volume excess and centrifugation, removal of subnatant and PBS dilution was repeated three times. Purified gas vesicles were quantified using the Micro BCA Protein Assay Kit (Thermo Fisher Scientific). Gas vesicles were collapsed with hydrostatic pressure before quantification. Bovine serum albumin was used to generate the standard curve. Absorbance measurements were taken on a Spectramax M5 spectrophotometer (Molecular Devices).

TEM sample preparation and imaging. Cells expressing ARGs, or purified gas vesicles, were exchanged into water or 10 mM HEPES pH 8.0 with 150 mM NaCl, respectively, via three rounds of buoyancy purification and buffer exchange as described above. Samples were deposited on Formvar/carbon 200 mesh grids (Ted Pella) that were rendered hydrophilic by glow discharging (Emitek K100x). For purified gas vesicles, 2% uranyl acetate was added for staining. The samples were then imaged on a FEI Tecnai T12 transmission electron microscope equipped with a Gatan Ultrascan CCD. Images were processed with Fiji35.

Hydrostatic collapse pressure measurements. Cells expressing ARGs, or purified gas vesicles, were diluted to OD600nm = 1.0 in PBS and 0.4 ml was loaded into an absorption cell (176.700-QS, Hellma GmbH). A single valve pressure converter (PC series, Alicat Scientific), supplied by a 1.5-MPa nitrogen gas source, applied hydrostatic pressure in the cell, while a microspectrophotometer (STS-VIS, Ocean Optics) measured the optical density of the sample at 500 nm. OD600nm was measured from 0 to 1.2 MPa gauge pressure with a 10-kPa step size and a 7-s equilibration period at each pressure.

In vitro ultrasound imaging. Phantoms for imaging were prepared by melting 1% (w/v) agarose in PBS and casting wells using a custom 3D-printed template. Cells at 2 × 10^7 cells/ml to 2 × 10^8 cells/ml after exposure to 1.2 MPa hydrostatic pressure to eliminate any contribution to light scattering from gas vesicles. The optical density was then converted into cells per ml using the relationship OD = 8.10 × 10^-10 cm^-1 (https://www.genomics.agilent.com/biocalculators/calcODBacteria.jsp). Cell samples collected at early time points following induction, which had an optical density insufficient for loading, were first concentrated using centrifugation at 350g. Ultrasound imaging was performed using a Verasonics Vantage programmable ultrasound scanning system and L22-14v 128-element linear array transducer (Verasonics). The transducer was mounted on a computer-controlled 3D translatable stage (Velmax). Image acquisition was performed using conventional B-mode imaging using a 128-raylines protocol with a synthetic aperture to form a focussed excitation beam. The transmit waveform was set to a frequency of 19 MHz, 67% intra-pulse duty cycle, and a one-cycle pulse. Samples were positioned 6 mm from the transducer face, which was covered with a 0.2% agarose layer to form a layer of PBS. The transmit beam was also digitally focused at 6 mm. For imaging, the transmit voltage was 2 V and the f-number was 3, resulting in a peak positive pressure of 0.4 MPa. Backscattered ultrasound signals were filtered with a 7-MHz bandpass filter centred at 19 MHz. Signals backscattered from four transmit events were summed before image processing. Pixel gain was set to 3 and persistence to 90. For gas vesicle collapse using the L22-14 array, we set the f-number to 0.2 (thereby ensuring that all transducer elements were active) and scanned the transmit focus from 3 mm to 9 mm. During the 10-s collapse scan, single-cycle pulses were applied using a ray-lines protocol at 19 MHz with a frame rate of 12 frames per second. To measure gas vesicle collapse in ARG-expressing cells as a function of acoustic pressure, images were acquired as described above at a peak positive pressure of 0.4 MPa after sequentially exposing the samples to collapse pulses of increasing amplitude, with pressures that varied from 0.55 MPa to 4.7 MPa. To achieve complete collapse, we applied the maximal pressure of 4.7 MPa. Collapse data were fitted with a Boltzmann sigmoid function to facilitate visualization of collapse curves. This function is of the form \( f(p) = \frac{a + \frac{b}{p}}{1 + \frac{p}{c}} \), where \( p \) is the pressure, and \( a, b, c \) and \( d \) are fitted parameters representing the collapse midpoint and slope, respectively. For spectral unmixing, the two collapse pressures applied were 2.7 MPa and 4.7 MPa. Transducer output pressures were measured in a degassed water tank using a fibre-optic hydrophone (Precision Acoustics).

Plate-based induction and optical imaging. ARG and GFP constructs were transformed as described above, and the transformation mix after recovery was plated on two-layer LB-Agar plates. The underlayer contained 50 μg/ml kanamycin, 1.0% t-arabinose, and 0.8 mM IPTG. The overlayer contained 50 μg/ml kanamycin and 0.4% glucose. The overlay was poured 30 min before plating, and each layer was 4 mm thick. Plates with transformants were incubated at 30 °C for 2 h and then imaged for white light scattering and green fluorescence using a Chemidoc MP instrument (Bio-Rad).

Cell growth, viability and microcin production assays. E. coli Nissle 1917 cells were transformed by electroporation with PET28 plasmids containing either the argl or lux gene cluster under the T7 promoter. Transformed cells were grown in 5 ml starter cultures in LB medium containing 50 μg/ml kanamycin, 1% glucose for 16 h at 37 °C. The overnight cultures were diluted 1:100 in 50 ml of LB medium containing 50 μg/ml kanamycin and 0.2% glucose. Cultures were grown at 30 °C to OD600nm ≈ 0.2–0.3 and induced with 3 mM IPTG (+IPTG), or left uninduced (–IPTG). Both induced and uninduced cultures were allowed to grow for 22 h at 30 °C. For time point optical density measurements, 1 ml of the culture was taken under measurement. For the final 22 h of growth, the cultures were diluted to a uniform OD600nm of 0.2, before further serial dilution by a factor of 2 × 10^3 in LB supplemented with 50 μg/ml kanamycin and 0.2% glucose. 100 μl of the final dilutions was plated on two-layer LB agar plates using a cell spreader. The under-layer of the plates contained 50 μg/ml kanamycin and 9 μg/IPTG. The overlayer contained 50 μg/ml kanamycin and 0.4% glucose. The overlayer was poured 30 min before plating, and each layer was 3 mm thick. Cells uniformly spread over the two-layer plates were allowed to grow at 30 °C for 21 h. Colonies were then imaged for light scattering using the Chemidoc MP instrument under white light illumination and 605 ± 50 nm receive filter, and both opaque (gas vesicleexpressing cells) and transparent (wild-type) cells were imaged. For forming units units per millilitre and the gas vesicle-expressing fraction. Plates had a minimum of 82 and a maximum of 475 total colonies, enabling manual counting. To assay microcin production, E. coli Nissle 1917 cells containing argl or lux were cultured as described above and spotted on microcin assay plates containing E. coli K-12 H5316 cells (gift from K. Hanke). Wild-type H5316 were grown in 5 ml LB medium, and H5316 cells transformed with PET plasmid containing mWasabi and KanR under a T5 promoter (H5316* cells) were grown in 5 ml LB medium containing 50 μg/ml kanamycin and 1% glucose for 16 h at 37 °C. Two-layer LB plates were used to assay the growth inhibition of H5316 cells by microcin peptides produced by Nissle 1917 cells. Plates were used to assay with wild-type H5316 cells containing 20 ml of 1% LB agar at the bottom, and the top layer contained 2 × 10^5 H5316 cells in 20 ml of 0.3% LB agar. Plates using H5316* cells contained 20 ml of 1% LB agar with 50 μg/ml kanamycin, 50 μg/mesalfer, and 3 μg/IPTG, and the top layer contained 2 × 10^5 H5316* cells in 20 ml of 0.3% LB agar with 50 μg/ml kanamycin, 50 μg/mesalfer, and 3 μg/IPTG. Nissle cells containing
arg1 or lux genes were cultured at 30 °C for 22 h with or without 3 μM IPTG. Nissile cells with arg1 were exposed to 1 MPa of hydrostatic pressure to facilitate the removal of kanamycin by centrifugation before spotting on H5316 plates. Nissile cells containing arg1 and lux induced and uninduced with IPTG, as well as H5316* cells, were washed 3 × in PBS by pelleting and adjusted to OD600nm = 1 in LB. All cells were spotted in 2-μl volume on 5-mm sterile filter paper (Bel-Art Products), placed on the microcin assay plates. Unsupplemented LB and 100 mM 1-ampicillin (2 μl each) were similarly spotted as controls. After 17 h at 37 °C, the plates were imaged with the Chemidoc MP instrument with blue transillumination, and unfiltered light was collected to form an image. Images shown are representative of four experiments each.

Colony ultrasound. ARG and GFP constructs were transformed into BL21(A1) one-shot competent cells (Thermo Fisher Scientific) and plated onto LB agar two-layer inducer plates as described above. Plates were grown at 37 °C for 14 h. The colonies were immobilized by depositing a 4 mm layer of 0.5% agarose–PBS gently onto the plate surface. Ultrasound imaging was performed using a L11-4v128-element linear array transducer (Verasonics) to obtain a larger field of view. The transducer was mounted on a computer-controlled 3D translatable stage (Veltem). Image acquisition was performed using conventional B-mode imaging using a 128-ray-lines protocol with a synthetic aperture to form a focused excitation beam. The transmit waveform was set to a frequency of 6.25 MHz, 67% intra-pulse duty cycle, and a four-cycle pulse. Colonies were positioned 20 mm from the transducer face, which is the elevation focus of the L11-4v transducer, coupled through a layer of PBS. The transmit beam was also digitally focused at 20 mm. For imaging, the transmit power was 2 V and the f-number was 3, resulting in a peak positive pressure of 0.61 MPa. To measure gas vesicle collapse in bacterial colonies as a function of acoustic pressure, images were acquired as described above at a peak positive pressure of 0.61 MPa after sequentially exposing the samples to collapse pulses at 6.25 MHz, with increasing amplitude from 0.61 MPa to 5.95 MPa. Pixel gain in the images was set to 0.1 and persistence to 20. Cross-sectional images of the plate (perpendicular to the plate surface) were acquired at spatial intervals of 250 μm using computer-controlled steps. The cross-sectional images were processed in MATLAB to form 2D images of the plate surface. First, the cross-sectional images were stacked to produce a 3D-volumetric reconstruction of the plate. We then summed the signals in a 2-mm slice of the volume parallel to and centred on the bacterial growth surface after thresholding to eliminate background, forming a 2D projection image of the plate. After ultrasound imaging, image processing, and acoustic phenotype prediction, the colonies were picked using 10-μl sterile pipette tips. Each colony was used to inoculate a 5-ml LB culture containing 50 μg ml−1 kanamycin culture. DNA was extracted from the cultures by mini-prep (PureYield, Promega) and sequenced to determine whether the plasmid contained GFP, arg1 or arg2.

In vivo ultrasound and bioluminescence imaging. All in vivo experiments were performed on BALB/c or SCID nude female mice, aged 14–15 weeks, under a protocol approved by the Institutional Animal Care and Use Committee of the California Institute of Technology. No randomization or blinding were necessary in this study. Ultrasound imaging was performed as follows. Mice were anaesthetized with 1–2% isoflurane, maintained at 37 °C on a heating pad, depicted over the imaged region, and imaged using an L22-14v transducer with the pulse sequence described above. For imaging of E. coli in the gastrointestinal tract, BALB/c mice were placed in a supine position, with the ultrasound transducer positioned on the lower abdomen, transverse to the colon. Anatomical landmarks including the bladder were used to identify the position of the colon. Prior to imaging, buoyancy-enriched E. coli Nissile 1917 expressing arg1 or lux were mixed in a 1:1 ratio with 42 °C 4% agarose–PBS for a final bacterial concentration of 107 cells ml−1. An 8-gauge needle was filled with the mixture of agarose and bacteria expressing either arg1 or lux. Before it solidified, a 14-gauge needle was placed inside the 8-gauge needle to form a hollow lumen within the gel. After the agarose–bacteria mixture solidified at room temperature for 10 min, the 14-gauge needle was removed. The hollow lumen was then filled with the agarose–bacteria mixture expressing the other imaging reporter (arg1 or lux). After it solidified, the complete cylindrical agarose gel was injected into the colon of the mouse with a PBS back-filled syringe. The same procedure was used with E. coli BL21 cells, except with the entire gel homogeneously composed of either arg2- or GFP-expressing cells. Introduction of gel into the colon is a common preparatory protocol for gastrointestinal ultrasound6,37.

For imaging of S. typhimurium in tumours, we formed hind-flank ovarian tumour xenografts in SCID nude mice via subcutaneous injection of 5 × 106 OVCAR8 cells (provided by the National Cancer Institute tumour repository with certificate of authentication) with Matrigel. After tumours grew to dimensions larger than approximately 6 mm (14 weeks), they were injected with arg1-expressing S. typhimurium (50 μl, 3.2 × 106 cells ml−1). The tumours were then imaged with ultrasound, with anaesthetized mice in a prone position (homeostasis and imaging parameters as described above). Our animal protocol specified that animals with total tumour volume exceeding 2 cm3, or showing signs of distress as assessed by the veterinary team, be euthanized.

For luminescence imaging, mice were anaesthetized with 100 mg kg−1 ketamine and 10 mg kg−1 xylazine and imaged using a Bio-Rad ChemiDoc MP imager without illumination, no emission filter, and an integration time of 5 min. The image was thresholded and rendered in ImageJ, and superimposed on a bright-field image of the mouse using GIMP.

Image processing. MATLAB was used to process ultrasound images. Regions of interest (ROIs) were defined to capture the ultrasound signal from the phantom wall, colon, or tumour region. All in vitro phantom experiments had the same ROI dimensions. For in vivo experiments ROIs were selected consistently to exclude edge effects from the colon wall or skin. Mean pixel intensity was calculated from each ROI, and pressure-sensitive ultrasound intensity was calculated by subtracting the mean pixel intensity of the collapsed image from the mean pixel intensity of the intact image. Images were pseudo-coloured, with maximum and minimum levels adjusted for maximal contrast as indicated in accompanying colour bars. For the multiplexed imaging of arg1 and arg2, acoustic spectral unmixing was performed as previously described36. In brief, a spatial averaging filter (kernel size 30 × 30 pixels or 750 × 750 μm) was applied to the three acquired images (before collapse, after collapse with 2.7 MPa and after collapse with 4.7 MPa) to reduce noise. Then, pixel-wise differences between the first and second image, and between the second and third image, were calculated, and multiplied by the inverse of the collapse matrix, α, representing the expected fractional collapse of each ARG type at each pressure (α = (0.7921, 0.5718, 0.2079, 0.4282)), to produce the unmixed pixel intensities corresponding to the contributions from arg2 and arg1.

Statistical analysis. For statistical significance testing, we used two-sided heteroscedastic t-tests with a significance level of type I error set at 0.05 for rejecting the null hypothesis. Sample sizes for all experiments, including animal experiments, were chosen on the basis of preliminary experiments to be adequate for statistical analysis.

Code availability. MATLAB code is available from the corresponding author upon reasonable request.

Data and code availability. arg1 and arg2 plasmid sequences are included in Supplementary Information, and plasmids will be available from Addgene. All other materials are available from the corresponding author upon reasonable request.

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Extended Data Figure 1 | Sequence homology of GvpA/B. Amino acid sequence alignment of the primary gas vesicle structural protein GvpB from *B. megaterium* (the GvpA analogue in this species) and GvpA from *A. flos-aquae*. 
Extended Data Figure 2 | Ultrasound contrast from buoyancy-enriched cells. a, Diagram of centrifugation-assisted enrichment of buoyant cells. b, Image of arg1 E. coli culture 22 h after induction and 4 h of centrifugation at 350 g, showing the presence of buoyant cells. Arrowhead points to the meniscus layer containing buoyant cells. Experiment repeated three times with similar results. c, Ultrasound images of E. coli expressing arg1 at various cellular concentrations, with and without buoyancy enrichment. Experiment was repeated three times with similar results. d, Ultrasound contrast from E. coli expressing arg1, with and without buoyancy enrichment, and GFP at various cell densities. Data are from three biological replicates; lines represent the mean.
Extended Data Figure 3 | Time course of acoustic reporter gene contrast after induction. a, Ultrasound images of arg1-expressing *E. coli* at various times after induction with IPTG. Experiment repeated four times with similar results. b, Ultrasound contrast at each time point. Data are from four biological replicates; line represents the mean. Cell concentration, $5 \times 10^8$ cells ml$^{-1}$. Scale bar, 2 mm.
Extended Data Figure 4 | Acoustic reporter gene expression and ultrasound imaging does not affect cell viability. a, Growth curves of *E. coli* containing the arg1 or GFP expression plasmid, with or without induction using 0.4 mM IPTG. Data are from three biological replicates per sample; lines represent the mean. b, Representative TEM images of whole *E. coli* cells expressing arg1 with and without exposure to acoustic collapse pulses, and *E. coli* cells expressing GFP. Images were acquired from three biologically independent samples for arg1, two for arg1 with ultrasound collapse and one for GFP (more than 50 cells imaged per sample) with similar results. c, Dark-field optical image of agar plate containing colonies of *E. coli* expressing arg1 14 h after seeding. d, Image of the same plate after the right half of the plate was insonated with high-pressure ultrasound. e, Image of the same plate 20 h after insonation. f, Image after the right half of the plate in e was insonated with high-pressure ultrasound. Zoomed in images of representative colonies shown below each plate image. Scale bars, 500 nm. Experiment was repeated three times with similar results.
Extended Data Figure 5 | Multiplexed imaging of genetically engineered reporter variants. a, Image of arg2 E. coli culture 22 h after induction showing the presence of buoyant cells (top). Experiment repeated three times with similar results. Mass fraction of gas vesicles produced 22 h after induction (bottom). Line represents the mean.

b, Ultrasound contrast from the whole population of cells expressing arg1, arg2 or GFP. Lines represent the mean. c, Ultrasound contrast from the buoyancy-enriched population of cells expressing arg1, arg2 or GFP. Lines represent the mean. d, Normalized optical density (representing the intact fraction) of gas vesicles isolated from E. coli expressing arg1 or arg2 as a function of applied hydrostatic pressure. e, Normalized ultrasound intensity as a function of peak positive pressure from 0.6 to 4.7 MPa for E. coli expressing arg1 or arg2. f, Acoustic collapse spectra derived by differentiating the data and curves in e with respect to applied pressure. a–f, Data are from three biological replicates per sample. d–f, Curves represent fits of the data using the Boltzmann sigmoid function to assist visualization.
Extended Data Figure 6 | Anatomical ultrasound images of acoustic bacteria in the gastrointestinal tract. Raw images underlying the difference maps shown in Fig. 4e, g. The cyan outline identifies the colon region of interest for difference processing. This experiment was repeated three times with similar results.
Extended Data Figure 7 | Ultrasound imaging of ARG-expressing cells in the mouse colon. a, Transverse ultrasound images of mice whose colon contains BL21 *E. coli* expressing either *arg2* or GFP at a final concentration of $10^9$ cells ml$^{-1}$. A difference heat map of ultrasound contrast within the colon region of interest before and after acoustic collapse is overlaid on a grayscale anatomical image. b, Signal intensity in mice with *E. coli* expressing either *arg2* or GFP. Data are from 5 biological replicates per sample. $P$ value = 0.02 using two-sided heteroscedastic *t*-test. Scale bar, 2 mm.
Extended Data Figure 8 | Effect of arg1 and lux expression on ECN cell growth, viability and microcin release. a, Optical density at 600 nm measured from 0 to 22 h after induction with 3μM IPTG, or without induction, in ECN cells transformed with arg1 or lux. Data are from four biological replicates per time point, lines represent the mean. For comparisons between induced arg1 and induced lux values at 22 h P = 0.12. For comparisons between uninduced arg1 and uninduced lux at 22 h P = 0.04. For comparisons at all other time points P > 0.14. b, Colony-forming units (cfu) per millilitre culture per OD 600nm after 22 h of induction with 3μM IPTG, or uninduced growth, of ECN cells transformed with arg1 or lux. P ≥ 0.22. Data are from 7 biological replicates for arg1 samples and four biological replicates for lux samples. Lines represent the mean. c, Fraction of opaque, gas vesicle-producing colonies produced by plating arg1-transformed ECN cells 22 h after induction with 3μM IPTG, or uninduced growth. Cells were plated on dual-layer IPTG induction plates, allowed to grow overnight at 30°C, and imaged as in (Extended Data Fig. 4c–f, P = 0.12. data are from seven biological replicates, lines represent the mean. d, Microcin release assay using a uniform layer of the indicator strain E. coli K12 H5316 in soft agar, after 17-h incubation with filters containing microcin sources and controls, as indicated. ECN cells transformed with arg1 or lux were induced for 22 h with 3μM IPTG, or grown without induction, before spotting. H5316* indicates H5316 cells transformed with mWasabi and cultured for 22 h as with ECN cells. All cells were washed before spotting to remove antibiotic. Experiment was performed four times with similar results. Amp, 100 mg ml⁻¹ ampicillin; LB, LB medium. e, As in d, but with the indicator strain comprising H5316* cells and the agar containing 50μg ml⁻¹ kanamycin, 3μM IPTG and 50μM desferal, to show that microcin release also occurs during transgene expression. Note that the H5316* spot appears bright because the plate image is acquired with blue-light transillumination, resulting in mWasabi fluorescence. Experiment was performed four times with similar results. All P values were calculated using a two-sided heteroscedastic t-test.
Extended Data Figure 9 | Ultrasound imaging of *S. typhimurium* in tumour xenografts. a, Diagram of tumour imaging experiment. *S. typhimurium* expressing *arg1* were introduced into the tumours of mice and imaged with ultrasound. b, Ultrasound images of a gel phantom containing *S. typhimurium* expressing *arg1* or the *lux* operon. Cell concentration is $10^9$ cells ml$^{-1}$. Experiment repeated three times with similar results. c, TEM images of whole *S. typhimurium* cells expressing *arg1* with and without exposure to acoustic collapse pulses. At least 20 cellular images were acquired for each sample type (from one biological preparation each) with similar results. d, Ultrasound images of mouse OVCAR8 tumours injected with 50μl of $3.2 \times 10^9$ cells ml$^{-1}$ *arg1*-expressing *S. typhimurium*, before and after acoustic collapse. Experiment repeated five times with similar results. e, Collapse-sensitive ultrasound contrast in tumours injected with *arg1*-expressing or *lux*-expressing cells. Data are from five animals, line represents the mean. $P = 0.002$ using a two-sided heteroscedastic $t$-test. Scale bars, 2 mm (b), 500 nm (c) and 2.5 mm (d).
Extended Data Figure 10 | High-throughput screening of acoustic phenotypes. a, Ultrasound intensity histogram of 22 randomly picked colonies. Colonies with low contrast were predicted to contain the gene encoding GFP and those with high contrast to contain genes encoding arg1 or arg2 genes. b, Normalized change in ultrasound intensity (U) for each of the 15 arg1 or arg2 colonies after insonation at increasing pressures. At 4 MPa, colonies with signal above the indicated threshold were predicted to be arg1 and below to be arg2. This experiment was performed once; each colony was treated as a biological replicate.
Experimental design

1. Sample size
   Describe how sample size was determined.

   Sample sizes for all experiments, including animal experiments, were chosen on the basis of preliminary experiments to be adequate for statistical analysis.

2. Data exclusions
   Describe any data exclusions.

   n/a

3. Replication
   Describe whether the experimental findings were reliably reproduced.

   Stated in figure captions.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.

   No randomization was necessary in this study.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.

   No blinding was necessary in this study.

   Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters
   For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

   n/a | Confirmed

   - The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
   - A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
   - A statement indicating how many times each experiment was replicated
   - The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
   - A description of any assumptions or corrections, such as an adjustment for multiple comparisons
   - The test results (e.g. P values) given as exact values whenever possible and with confidence intervals noted
   - A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
   - Clearly defined error bars

   See the web collection on statistics for biologists for further resources and guidance.
Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.

MATLAB used for data and image analysis. MATLAB, Excel and Prism used for fitting and plotting.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

All materials and data available on reasonable request. ARG1 and ARG2 plasmids will be deposited to Addgene.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

n/a

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.

n/a

b. Describe the method of cell line authentication used.

n/a

c. Report whether the cell lines were tested for mycoplasma contamination.

n/a

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

n/a

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.

Information included in the “In vivo ultrasound and bioluminescence imaging” section of the Methods

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.

n/a