Supplemental Materials:

METHODS:

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Supplementary Table 1. Lysogeny Broth (LB) medium was used for all imaging experiments. All cultures for imaging and phenazine extraction were inoculated from early stationary phase cultures at an OD_{500} of 0.001 in 50 ml of LB and incubated shaking at 30°C overnight in 250 ml baffled flasks. When the large cultures reached early stationary phase (OD_{500} between 2.1 and 2.5), both imaging samples and samples for extraction were collected. For imaging, 1 ml of the culture was pelleted by micro-centrifugation, resuspended in 50 ul of 1x PBS, and imaged as described below. For phenazine extraction, 30 ml of culture was pelleted by centrifugation at 5000xg for 10 min, the supernatant was decanted and the pellet and supernatant were frozen separately at –80°C. Frozen samples were processed at a later time as described below. Because preliminary experiments suggested that only reduced phenazines were associated with the cells, care was taken to disrupt the cells as little as possible to avoid altering the redox state of the phenazines and therefore the phenazine concentration measured by extraction or imaging.

Phenazine extraction and quantification. For phenazine extractions, the frozen pellets were thawed, resuspended in 3 ml of 1x PBS, and sonicated on ice. The cell lysate sample was divided and 1.5 ml samples were extracted for pyocyanin or PCA. Additionally, 1.5 ml of the culture supernatant was also extracted for each phenazine. For pyocyanin, the sample was first extracted with chloroform and subsequently extracted by 0.01 N HCl. The sample for PCA extraction was first acidified by addition of 50 ul of 6 N HCl and then extracted with ethyl acetate followed by 1 M NaOH. For both

phenazines, standard curves were extracted in parallel and all extraction steps were well vortexed and incubated while shaking for at least 30 min to maximize extraction efficiency.

The phenazine concentrations in the cell extract and the supernatant were measured with respect to the extracted standard curve using a Biotek Synergy plate reader. Acidic PYO was measured at 520 nm and basic PCA was measured at 367 nm and both were compared to the baseline reading at 700 nm.

Two-Photon Microscope Set-up. Two-photon imaging took place in a custom-made system. Samples were excited by focusing a pulsed laser beam generated by a MIRA 900F titanium-sapphire laser pumped by a Verdi 10 laser (Coherent Inc.) using a 40x C-Apochromat 1.2 NA water immersion objective lens (Carl Zeiss Microimaging). A 3D image of the specimen was acquired by raster scanning the focus point inside the specimen using a computer-controlled pair of scanner mirrors (6350 Cambridge Technology) and a piezoelectric objective actuator (P720, Physik Instrumente). The emitted light is collected via the epi-luminescence path in a de-scanned configuration. The signal is separated from the laser beam via a dichroic mirror (675DCSPXR, Chroma Technology), filtered via a short-pass filter (E625SP, Chroma Technology), spectrally resolved in a spectrograph (MS125 Newport Corp.) equipped with a ruled grating (77414 Newport Corp.), and detected by a 16-channel photomultiplier tube (R5900U-01, Hamamatsu Photonics). The PMT output is processed (amplification, discrimination, single photon counting) and transmitted to a control PC by custom-made electronics (*21*). For bacteria imaging, the laser beam wavelength was 780 nm, the laser power delivered to the sample was 7 mW, and the pixel sampling time was 125 µsec. For pure compound imaging, the laser beam wavelength was tuned between 740 and 890 nm, the laser power delivered to the sample was 1 mW, and the pixel sampling time was 1

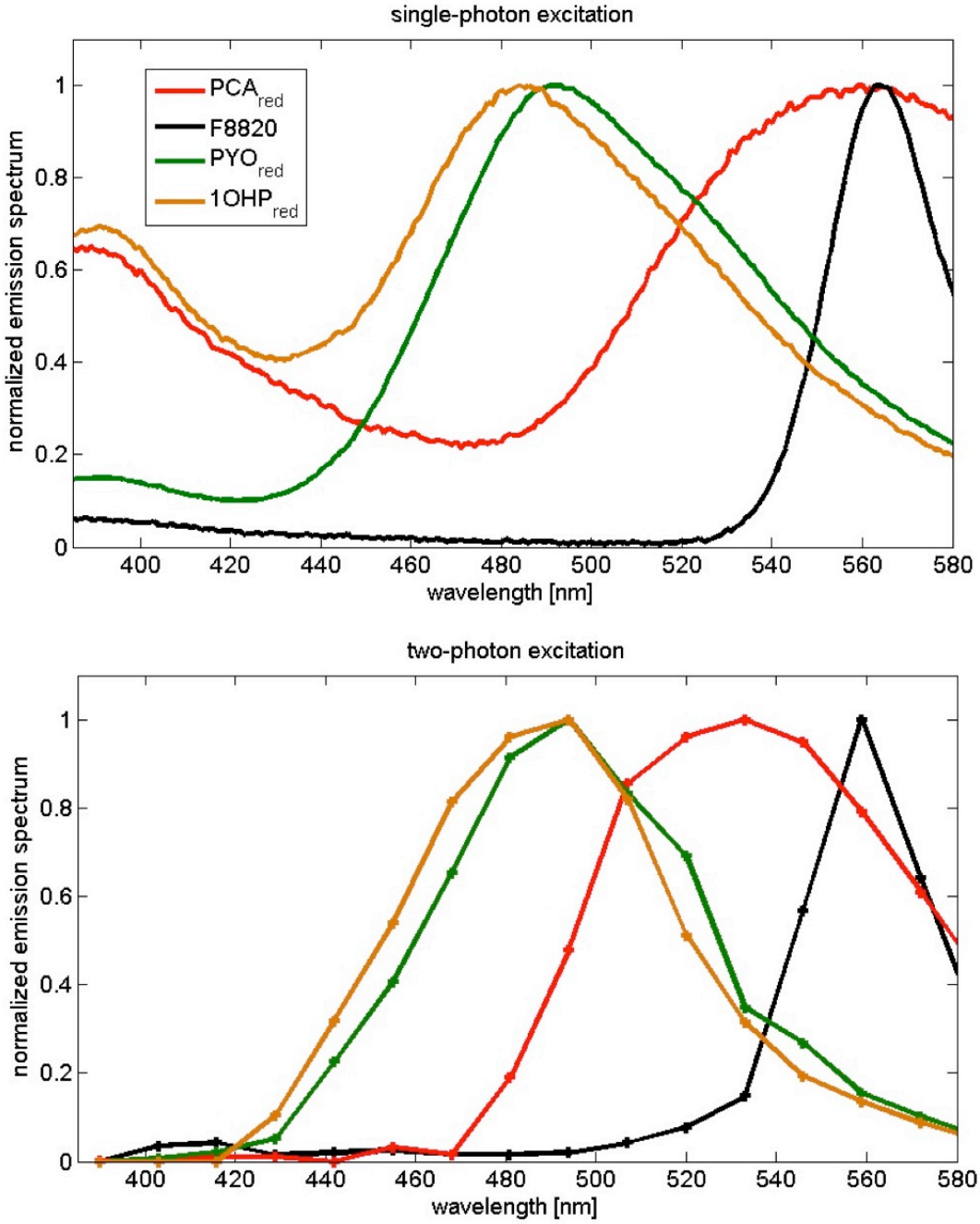
msec. All images digitized a 25×25 μm field of view into 256×256 pixels, 16 channels per pixel. Each PMT channel counted photons within a 13 nm-wide range of the spectrum, so that the 16 channels of the PMT cover the range [390, 600] nm.

Supplementary Table 1:

Strains used in this work:

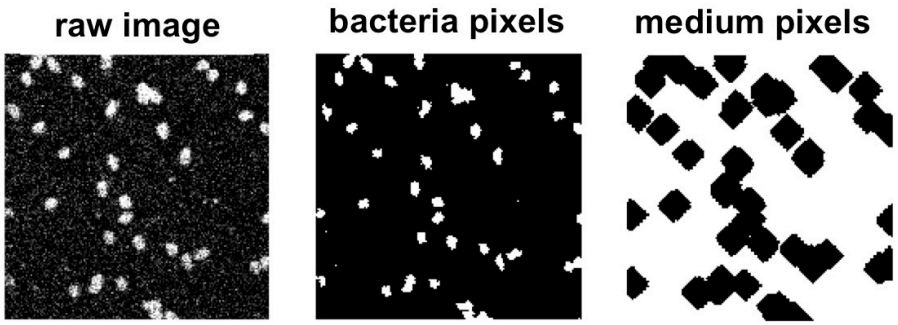
Strain name	Genotype	Source (Reference)
DKN263	PA14 (wildtype)	(*22*)
DKN330	PA14 Δ*phz*A1-G1, Δ*phz*A2-G2	(*17*)
LD375	PA14 Δ*phz*M	L. Dietrich, Columbia University
DKN619	PA14 Δ*pvdA,* Δ*pchE*	(*23*)
DKN620	PA14 Δ*phz*A1-G1, Δ*phz*A2-G2, Δ*pvdA,* Δ*pchE*	(*23*)

Supplementary Figure 1: Comparison of the emission spectra of PYO_{red}, PCA_{red}, $1OHP_{red}$ phenazines and F8820 fluorescent microspheres (Invitrogen) after single-photon (top) and two-photon excitation (bottom). Although the emission spectra of PYO_{red}, $1OHP_{red}$ and F8820 do not depend on the mode of fluorescent excitation (single-photon or two-photon), the emission spectrum of PCA_{red} is blue-shifted approximately 20 nm after two-photon excitation compared to single-photon excitation. The emission peaks of PCA_{red} and F8820 are approximately the same after single-photon excitation, however they differ significantly after two-photon excitation. The x-axis in the single-photon measurement corresponds to the exact wavelength of the measurement. The x-axis in the two-photon measurement shows the center wavelength of each one of the 16 sensor channels (each channels detects photons in a 13 nm range of the spectrum).



Supplemental Figure 1

Supplementary Figure 2: Example of a raw image (sum of the 16-channel raw data) and its segmentation into bacteria and medium pixels. Segmentation was based on the known noise properties of the sensor and a halo surrounding the bacteria was excluded from both groups of pixels.



Supplemental Figure 2

References:

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23. Wang, Y., Wilks, J.C., Danhorn, T., Ramos, I., Croal, L., and Newman, D.K. (2011) Phenazine-1-carboxylic acid promotes bacterial biofilm development via ferrous iron aquisition, *J. Bacteriol.*, in press.