SUPPLEMENTAL MATERIAL FOR: Single-Cell Analysis Reveals that the Enterococcal Sex Pheromone Response Results in Expression of Full-length Conjugation Operon Transcripts in All Induced Cells

Rebecca J. B. Erickson,[a] Arpan A. Bandyopadhyay,[b] Aaron M. T. Barnes,[a,c] Sofie A. O'Brien,[b] Wei-Shou Hu,[b] Gary M. Dunny[a]

[a]Department of Microbiology and Immunology, University of Minnesota, Minneapolis, Minnesota, USA, [b]Department of Chemical Engineering and Materials Science, University of Minnesota, Minneapolis, Minnesota, USA, [c]Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, Minnesota, USA

SUPPLEMENTAL TEXT

Not all induced cells appear to transfer by fluorescent reporter analysis of plasmid induction and transfer. Fluorescent reporters were designed to examine functional conjugation by induced cells at the single cell level (1). Microscopic tracking of the fluorescent expression of individual cells after mixing of donors and recipients embedded in agar pads allowed observation of donor induction and in some cases, formation of new transconjugants cells via transfer of the plasmid from an induced donor to a recipient cell as shown in the main paper (Fig. 2). Figure S1 shows a much larger field that was imaged as a merge of red, blue, and green fluorescence after 330 minutes of incubation of the embedded mating mixture. It is readily apparent that the vast majority of the donor cells are surrounded by recipients, with a significant fraction of this population appearing to be induced (green or yellow cells). However, many

uninduced cells (red) surrounded by recipients remained at the end of the experiment. This may be due to relatively low levels of **C** production in the embedded recipient cells,

Quantification of induction and conjugation on larger populations of mixed donor and recipient cells by flow cytometry (Fig. S4) also suggested that only a fraction of induced donors transferred the plasmid, even when recipients were in the majority (1). The fluorescent reporter is fused to early Q_{Op}, so it is possible that some of the cells that failed to transfer did not express all of the required conjugation proteins because they lacked full length Q_{Op} transcripts. However, this is unlikely, as we tested that the pCF10 derivative containing fluorescent reporters showed very similar transfer frequencies to those of wild type donors in standard liquid or plate matings.

SUPPLEMENTAL METHODS

Fluorescent protein reporter strain construction and Microscopic analysis were described in the main paper).

Flow cytometric analysis of donor induction and conjugation in liquid matings. Overnight cultures of *E. faecalis* donors (OG1RF+pCF10_tdTomato_iGFP) and recipients (OG1RF_CFP) were diluted 1:10 in fresh medium and grown to exponential growth phase without antibiotics for 1h at 37 °C. Donors were induced with 10 ng/ml of the ***C*** pheromone followed by 1h incubation at 37°C. The donors and recipients were then mixed in 1:10 ratio and mating was carried out at 37 °C for 2h. The mating suspensions were fixed using 2% (w/v) PFA for 10 mins at 4 °C. The bacterial clumps were broken up by EDTA treatment (100 mM), followed by Proteinase K

47 treatment (50 µg/mL, 10 mins at 55 °C). The cell suspension was further sonicated for
48 10 secs at 20% amplitude. The mating suspensions were then analyzed by flow
49 cytometry using a Fortessa X-20 flow cytometer. Expression of CFP, GFP, and
50 tdTomato was measured for 100,000 - 500,000 cells using the 405, 488, and 561 nm
51 laser excitation lines respectively. The cells were gated and analyzed using FlowJo v8
52 (Tree Star, Ashland, OR).

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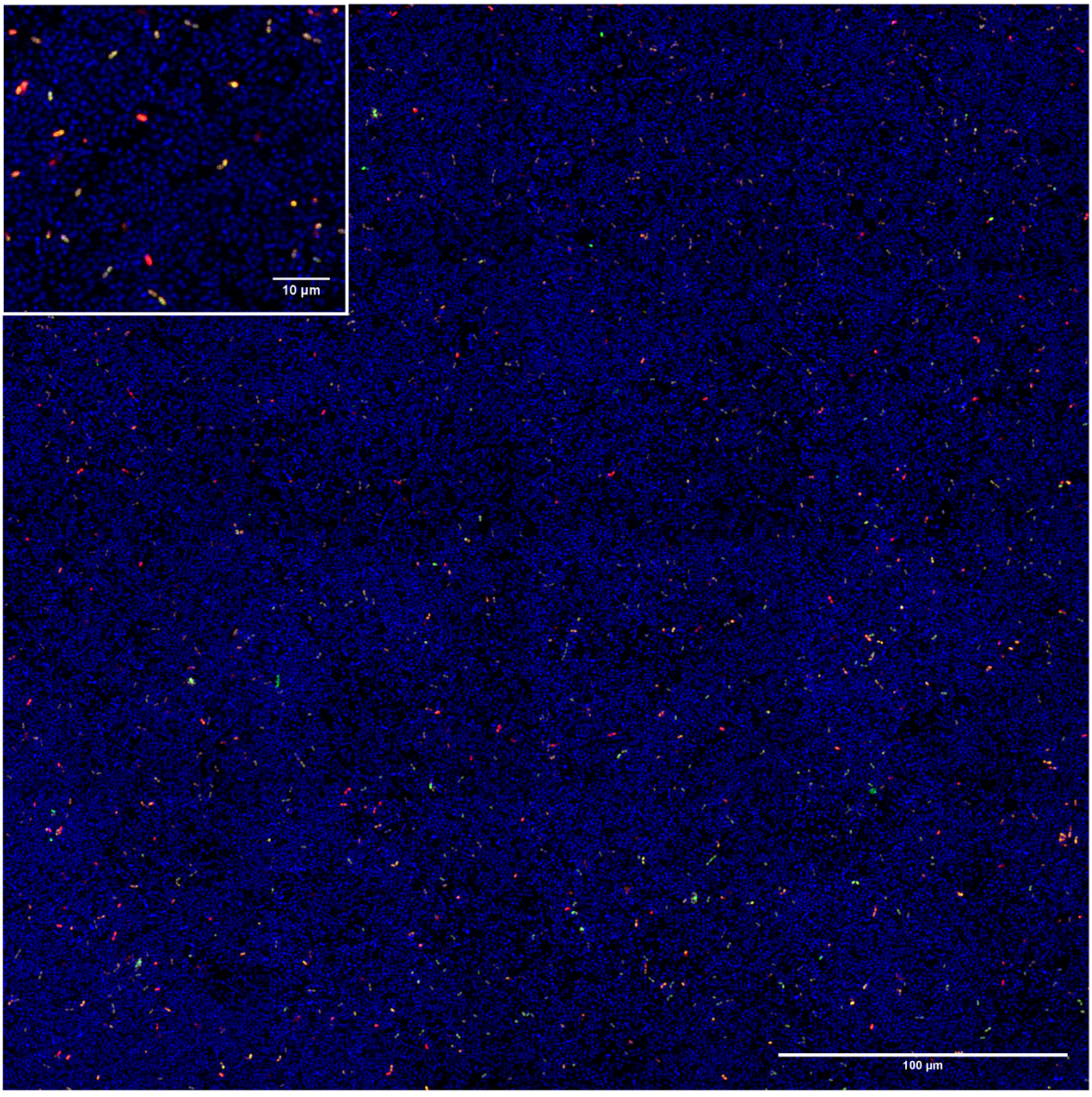
Supplemental figures / legends

FIGURE S1: Visualization of induction and conjugation via fluorescent reporter protein expression.



A lawn of *E. faecalis* cells on the surface of the agarose pad. This image is composed of 16 tiled image stacks stitched together (total area: 375µm × 375µm); a small portion of this image is also shown in Fig. 2. Recipients (blue; Hoechst 33342) and uninduced

donors (red; constitutive tdTomato expression) were mixed (D:R=1:100) and sandwiched between an agarose pad and coverslip-bottomed Petri dish. Donors express GFP (green) when induced by ***C***. The entire field was tracked every 30 mins for 6 hours using time-lapse laser-scanning confocal microscopy; t=330 min shown here. Note the relative paucity of induced donors relative to uninduced donor cells even though virtually all of the plasmid-carrying cells (td Tomato positive and/or GFP-positive; red, green or yellow in this image) are in close proximity to numerous recipients (Blue).

FIGURE S2: GFP expression increases ~300-fold after pheromone induction. Uninduced or induced (10 ng/ml **C** for 60 minutes) exponential phase donor cultures were treated to disperse large clumps, and subjected to flow cytometry as described in the supplemental methods. Nearly all cells express robust red fluorescence, indicative of carriage of pCF10. A. The image demonstrates the lack of green fluorescent cells in uninduced donor populations. B. The image demonstrates the fidelity of the GFP tag as a reporter of induction.

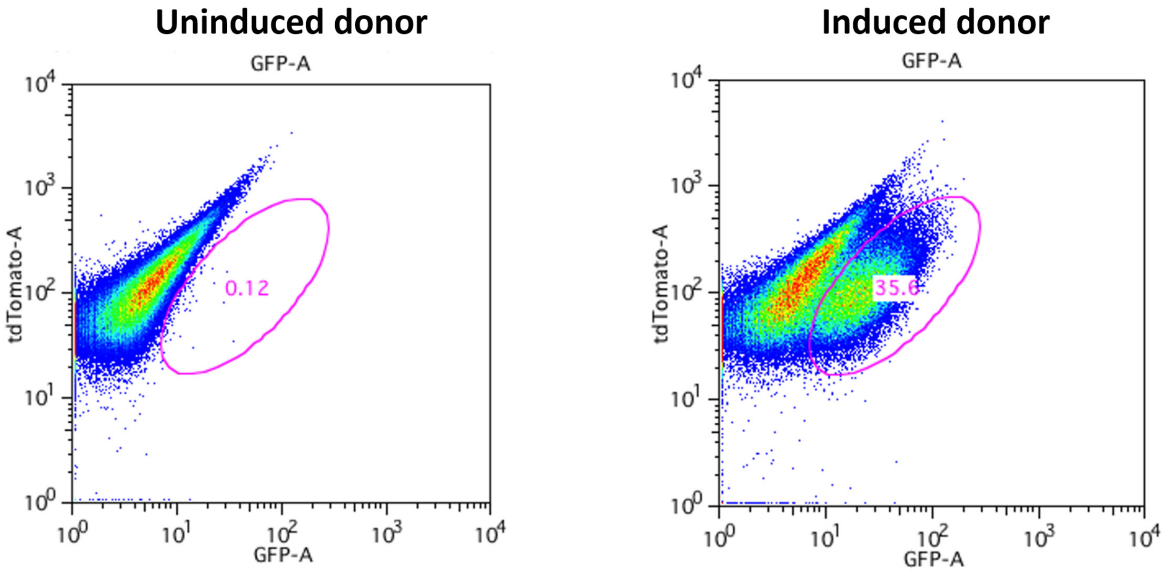
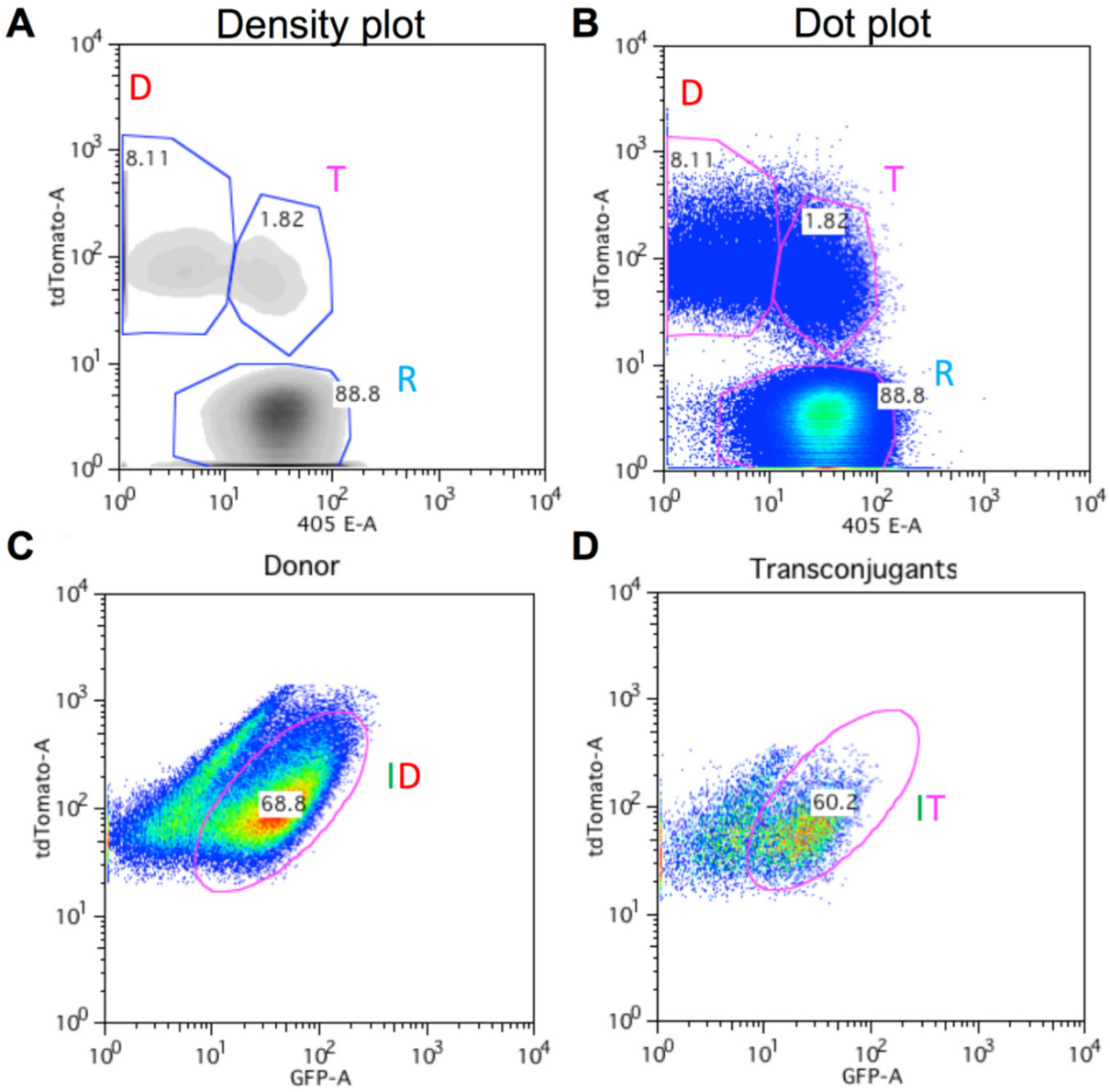


FIGURE S3: Results of flow cytometry-based analysis of fluorescent protein reporters of induction and conjugation.



(A) Two-parameter fluorescence density plot. (B), (C), and (D) Two-parameter fluorescence dot plots. (A-B) Three distinct populations can be identified from differential fluorescence patterns in the density and dot plots, D: donors, R: recipients, and T: transconjugants. The donor: recipient ratio is approximately 1:10. Although over 60% of donors were observed to be induced (C) and recipients were in excess,

transconjugants only make up about 1.8% of the total population (A) and (B). (D) About 60% of transconjugants were also observed to be induced.

Supplemental Tables

Supplemental Table S1: HCR probe sequences

HCR probes	Sequence (portion of probe homologous to transcripts of interest)	Amplifier code
Short Q_L-0	GCATTGAATTATTCCACAAGCGGGTCATTTTTAGAAAATGAGCGTGCTTG	**B4**
Short Q_L-1	CACGTTGTTTGCACGGCTCTTACGAGTAGTTCCAGTACAAACAATGCGTG	
prgA-1	TCAGGTGTTTTTGGTTGCGCTTGTTCTGCTGCCTGTACTTCATTTCCCCC	**B1**
prgA-5	TCCACGACTTTTTTAGCTTCGTCTACAACTGCTTGTTGGTCAGTCACTGC	
prgA-16	GATTTCATGCTTGCCACTTCGCCGTTTTGGTATTGACTACTATCCTCCAG	
prgA-21	CTTTCAGCACTTTAGCAGACGTTTGTTCAATCGCTTGTGCTTCCGCAACG	
prgA-23	CATAGGCTTCTTGTGCTTGAACCACTGCCGCTTCTGCTTTGTTTAACTCG	
prgB-3	TTCGCTTCATTTTCAGCTGGTGCAACTTCTGTTGGCTGCCCTAGAGGTTC	**B2D1**
prgB-5	TTGAACGCATTGTTGGCCCCAAAATCAGAAACAAATTCCGCATGGCCACC	
prgB-8	CCCCATTGCTAAAACAACAGAGCCGTCCGCGTTGGTTTTCATCATTGCTG	
prgB-16	TACGCTAGAATAAAGGCTTGTGGGTCTTTGGCAGAAATCGTCACCGTGCC	
prgB-22	TGGCTCTACTGGTGCTTGCGGTGTTTTTGGTGTTGGTGGTACAATCACTG	
prgJ-3	GACGGTAGTTATCTAAGTGATCTCCCTGATACGCCAATAAAACGTCTTCC	**B3**
prgJ-6	GTCCGTGGCGCAAAAAATAGCTGAACAAAGACAATCGCTCTAAGCCAGTC	
prgJ-17	CGCCATTCCTTTACCAGAACCAGACGTTCCGAAAATAAGCCCACTTGGTG	
prgJ-21	CGGTCAACAAGTCCCGCATCCACGTCCGTATAGCTTTTTAGTAGCGATTC	
prgJ-27	TTGCCCCGCTTTACTGTCTAATAACGTCGTAGGGTTTTGCGTGATACCTG	
pcfC-7	GTCCAATCTCTGGCAATATCCCACCGTCTGGGTCTGTTGTGATAAAGCTG	**B4**
pcfC-10	TCGTCCCCGGTCGTTTTTTCCACTTCTTTTGTTGCTTCAGTGACAGCTTC	
pcfC-15	TCATGGTCAAAAACAGAGTAACGTGCAGCAGCTATCCCTAGAACGCTGGC	
pcfC-26	AAACCAGACACTCGTCTCCTCCAATAAGCGCAACTTCACTCCTGTCAATC	
pcfC-27	TCATCAGTCGGTCCTGTTGCTAGTTCATCTGCTCGGTCATGCTGAAAAGC	
pcfG-4	TCTGATTTTGGATCGAATGTGTACCCGATTTTTCGTTGAATTGCTGCTTG	**B5**
pcfG-18	CCTTATACCCTCCTACAAAGAGCTTTCCAGAATTGCCATAGCCATAATCG	
pcfG-21	TCACAGGTTCTCTTTTCAGTGGTGTTTGTCCATTATACAGTCGTAATTGT	
pcfG-24	GCTCTAACATCTTCTCGTCAAGAGTCTCTAAAGTTTTTTCTGCTTCAAGA	
pcfG-29	AGTTTGGGCTTAATGTCGGTTTGCTTCTCTGGCTCTTTTTCCTGTACGGC	

Supplemental Table S2: HCR amplifier and fluorophore details

Transcript label	Amplifiers : Fluorophore
Short Q_L	B4H1, B4H2 : Alexa Fluor (AF) 647
prgA	B1H1, B1H2 : AF 488
prgB	B2H1, B2H2 : AF 488
prgB	B2H1, B2H2 : AF 647
prgJ	B3H1, B3H2 : AF 488
pcfC	B4H1, B4H2 : AF 488
pcfC	B4H1, B4H2 : AF 647
pcfG	B5H1, B5H2 : AF 488

Supplemental Table S3: Flow cytometry settings (A) and compensation matrix (B) used for analysis shown in Fig. 3.

A.

Flow cytometry parameter	Function	Voltage
FSC-A	Cell size	444
SSC-A	Cell granularity	253
Alexa Fluor 488	HCR-labeled transcripts	450
Alexa Fluor 647	WGA lectin (cell envelope)	135

B.

	Alexa Fluor 488	Alexa Fluor 647
Alexa Fluor 488	100.00	0.00
Alexa Fluor 647	8.59	100.00

SUPPLEMENTAL REFERENCES

1. Bandyopadhyay AA. 2018. Systems analysis of pheromone signaling and antibiotic resistance transfer in *Enterococcus faecalis*. Ph.D. thesis. University of Minnesota. http://hdl.handle.net/11299/197623