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Supplemental Information

**Anaerobic Bacteria Grow within
Candida albicans Biofilms and Induce
Biofilm Formation in Suspension Cultures**

Emily P. Fox, Elise S. Cowley, Clarissa J. Nobile, Nairi Hartooni, Dianne K. Newman,
and Alexander D. Johnson

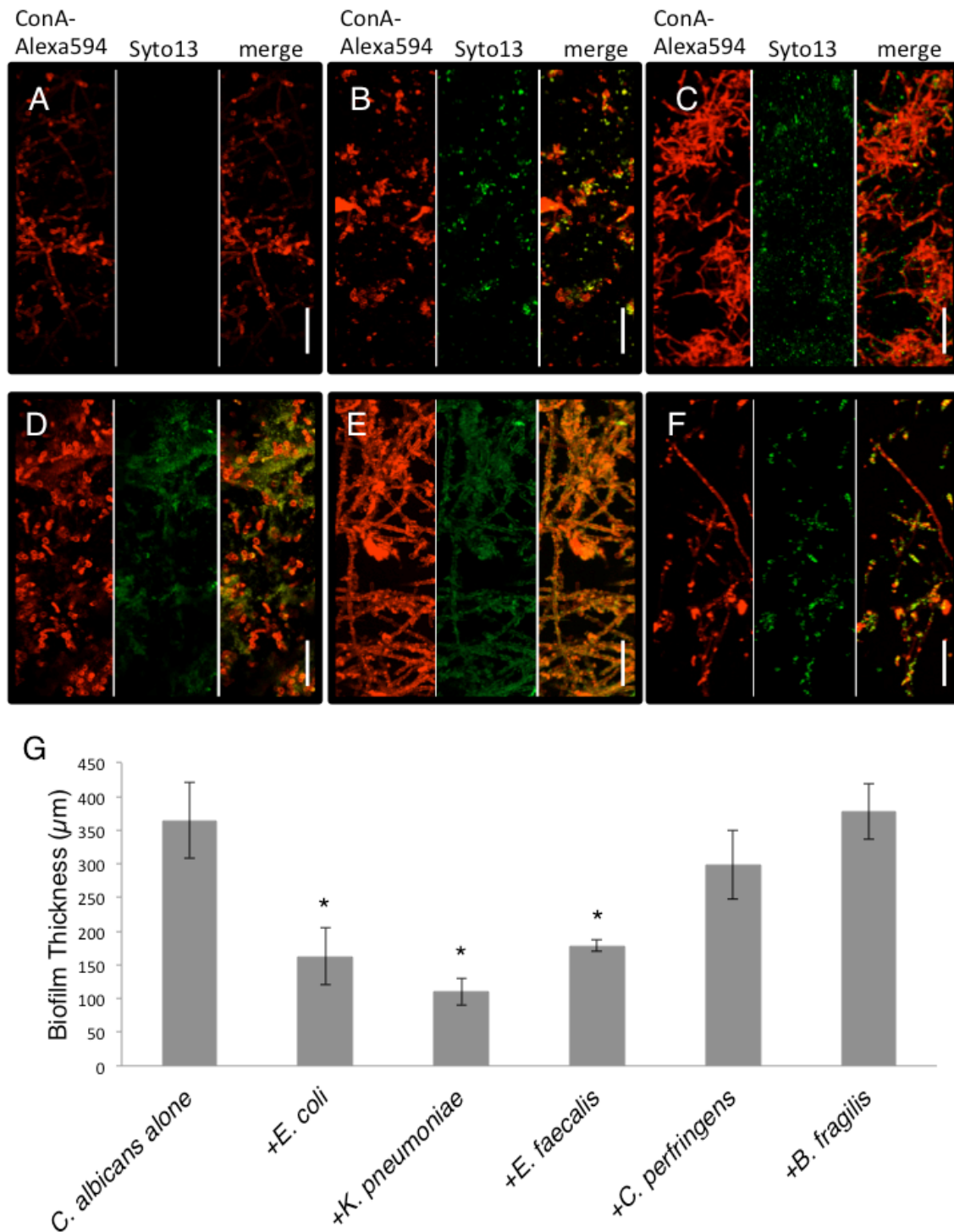


Figure S1, Related to Figure 1. Bacteria are incorporated throughout the biofilm. *C. albicans* was grown in biofilms for 24 hours either alone (A), or with *E.*

coli (B), *K. pneumoniae* (C), *E. faecalis* (D), *C. perfringens* (E), or *B. fragilis* (F). Biofilms were stained with conconavalin A – Alexa 594 and Syto 13 dyes, then imaged by CSLM. Images shown are top view, maximum intensity projection z-stacks of 65 μm thick sections internal to the biofilm. Scale bars are 50 μm . G) Biofilm thickness of at least six technical and two biological replicates was measured using CSLM. Average values shown, error bars indicate standard deviation. *Significantly different from *C. albicans* alone, $P < 0.001$, by student's paired t-test.

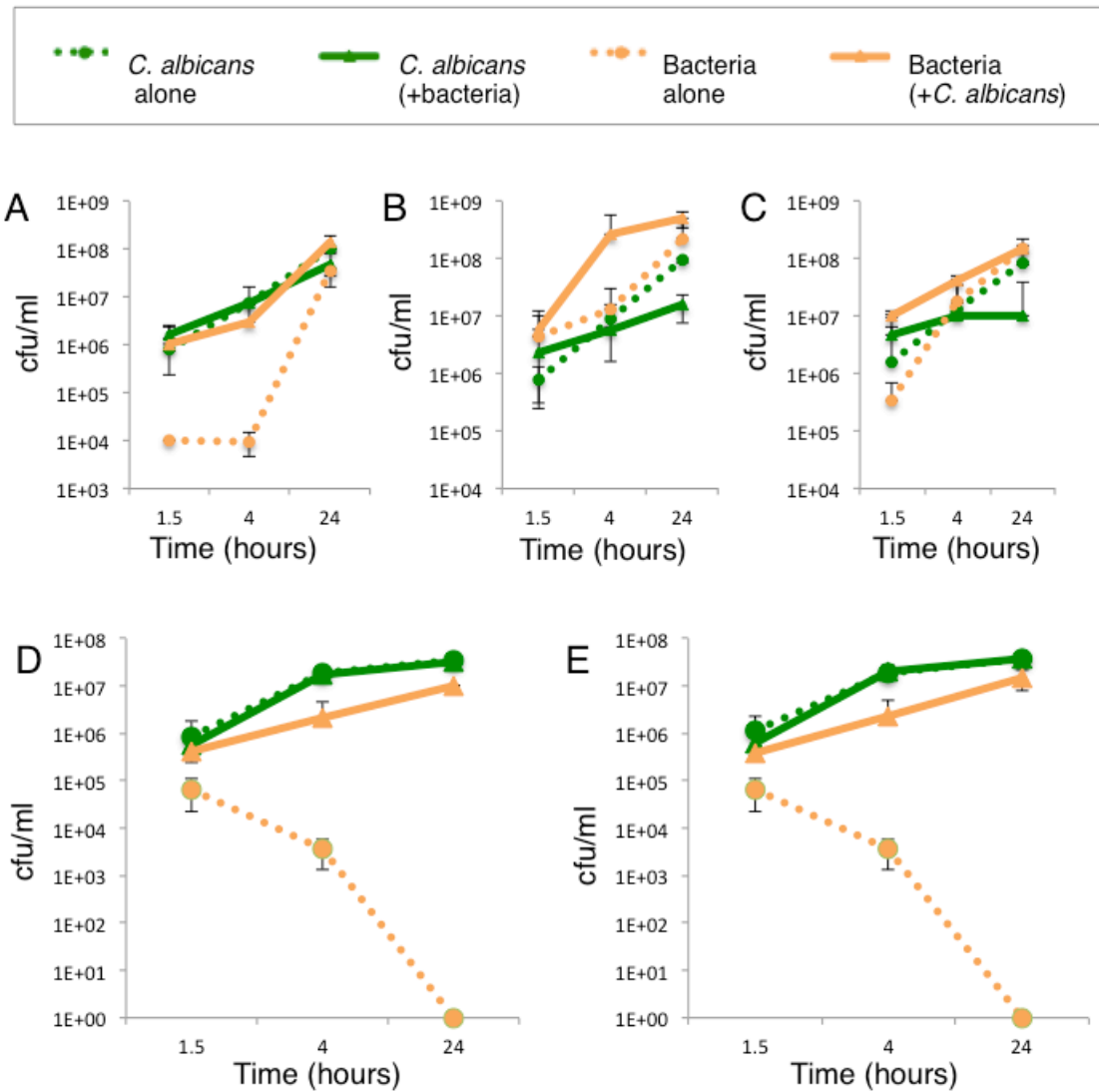


Figure S2, related to Figure 2. Incorporation of *C. albicans* and bacteria in biofilms is affected by co-culture. Measurement of cfu/ml of indicated species grown in biofilms in monoculture or co-culture, in biofilms in ambient oxic conditions. A) *C. albicans* SN250 and/or *E. coli*. B) *C. albicans* SN250 and/or *K. pneumoniae*. C) *C. albicans* SN250 and/or *E. faecalis*. D) *C. albicans* P57055 and/or *C. perfringens*. E) *C. albicans* CEC3494 and/or *C. perfringens*. Shown is the mean of at least two replicates, error bars are standard deviation.

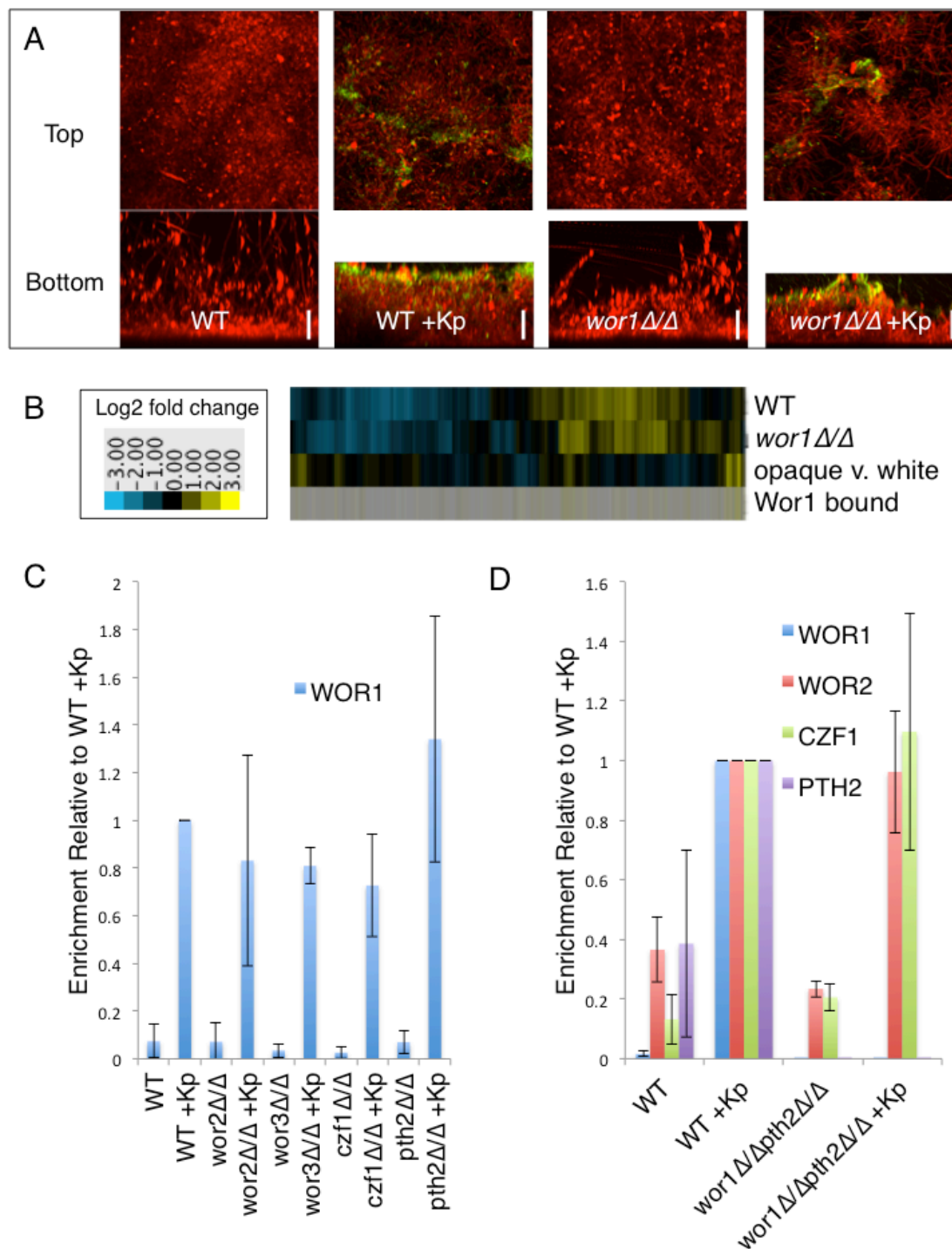


Figure S3, Related to Figure 3. *WOR1* is regulated independently from other opaque regulators during biofilm co-culture. A) Confocal Scanning Laser Microscopy of wild type (SN250) or *wor1*Δ/Δ (TF176) strain co-cultured with or

without *K. pneumoniae* (abbreviated Kp). Images shown are maximum intensity projections of the top and side view. Scale bars are 50 μm . B) Top two rows: heat map of gene expression in *C. albicans* (SN250) when co-cultured with *K. pneumoniae* in biofilms, compared to *C. albicans* alone. Values are the median values of at least two biological replicates. Bottom two rows: heat map of gene expression in opaque (AHY136) compared to white cells (AHY135); and genes whose promoters are bound by Wor1, data from [S1]. Along the x-axis are 6,111 genes. Yellow genes are upregulated or bound by Wor1, Blue genes are downregulated, gray genes are not bound by Wor1. C-D) Quantitative RT-PCR. Shown are the mean values of at least two biological replicates. Error bars are standard deviation. WT = SN250, Kp = *K. pneumoniae*. C) Expression of *WOR1* in the indicated strains. D) Expression of *WOR1*, *WOR2*, *CZF1*, and *PTH2* in the indicated strains.

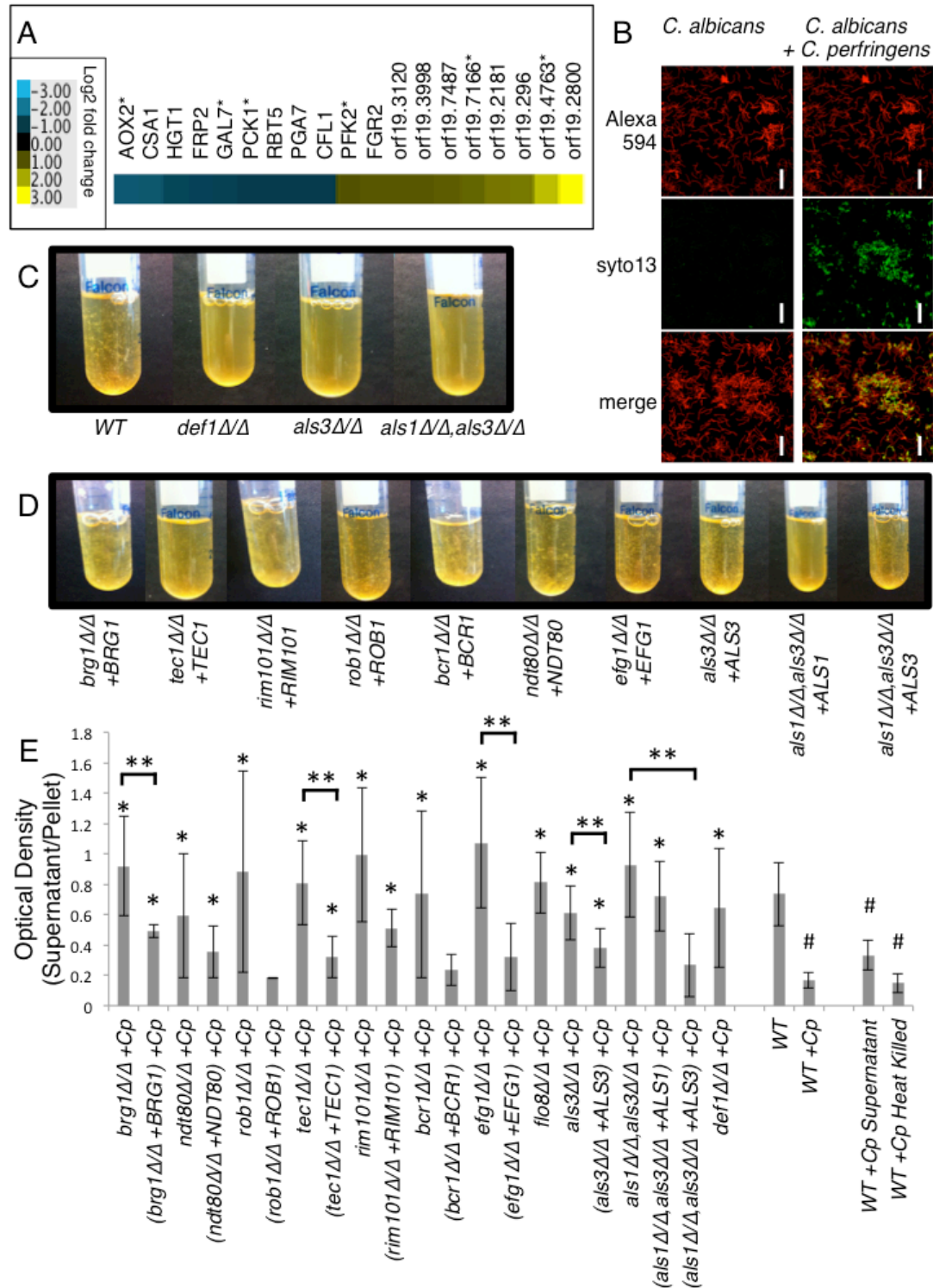


Figure S4, Related to Figure 4. Characterization of *C. perfringens*-induced aggregates when co-cultured in ambient oxic cultures with *C. albicans*. A) Heat map of gene expression in *C. albicans* when co-cultured in suspension with *C.*

perfringens, compared to *C. albicans* alone. Shown are genes differentially regulated more than two fold. Values are the median values of at least two biological replicates. Upregulated genes are yellow, downregulated genes are blue. *Genes that are regulated during hypoxia in *C. albicans*; these genes are significantly enriched in our dataset with a $p < 1.5 \times 10^{-5}$ by the chi-squared test. B) *C. albicans* was grown with or without *C. perfringens*, in biofilms for 1 hour. Biofilms were stained with conconavalin A – Alexa 594 and Syto 13 dyes, then imaged by CSLM. Images shown are maximum intensity projections of the top view. Scale bars are 50 μm . At least two replicates were visualized, representative images are shown. C - E) Suspension cultures of the indicated *C. albicans* strains, with or without *C. perfringens*, grown for 4 hours at 37°C. Assay was performed at least twice for each condition or mutant strain. C) *C. albicans* wild type or indicated mutant strains. D) *C. albicans* complemented strains where a wild type copy of indicated gene is restored in the deletion strain background. E) Aggregation was measured using an adapted assay from *S. cerevisiae*, in which flocculation is associated with greater sedimentation of aggregates, as measured by optical density (OD₆₀₀). Assay was performed at least twice, error bars are standard deviation. *Cp* = *C. perfringens*. *Significantly different from *WT + Cp*, $P < 0.05$. #Significantly different from *WT* alone, $P < 0.05$. **Significantly different between indicated deletion strain and complemented strain, $P < 0.05$. Student's paired t-test was used to calculate significance.

Supplemental Text

Wor1 is upregulated independently from other opaque-enriched transcription regulators during biofilm co-culture

To explore the role of *WOR1* in co-culture, we used a *wor1Δ/Δ* strain in biofilm co-cultures with *K. pneumoniae*. We found no morphological perturbations when biofilms were viewed by CSLM, and gene expression microarrays revealed few changes in the transcriptional profile induced by co-culture with *K. pneumoniae* (Figure S3A, B). It is known that in opaque cells, Wor1 is part of a closely intertwined transcriptional regulation network, where it regulates and is regulated by several other regulators. Both co-culture with *K. pneumoniae* and switching from white to opaque increases expression of regulators *WOR1*, *WOR2*, *WOR3*, and *CZF1*, and so we examined the connections between *WOR1* expression and the expression of other white-opaque regulators in co-culture with *K. pneumoniae*. We used RT-qPCR and the tiling probes in our arrays that cover *WOR2*, *WOR3*, and *CZF1* to determine whether expression of any of these regulators depend on Wor1, and whether expression of *WOR1* depends on any of these regulators, and found that they are independently induced (Figure S3C, Dataset 1, 2). *WOR1* expression is also independent of Wor2, Wor3, and Czf1 in white and opaque cells, and it is impossible to assay the effect of *WOR1* deletion in opaque cells on expression of the other regulators, because *wor1Δ/Δ* cells are locked in the white state [S1]. Therefore the independent nature of the induction of these regulators in co-culture does not necessarily distinguish it from the regulatory circuitry used in the white-opaque switch.

We found that expression of *PTH2* was induced by co-culture with *K. pneumoniae* (Figure 3B), which is interesting because *PTH2* is a homolog of *WOR1*, and has the same DNA binding domain, but the deletion strain has no known phenotype and no previously identified condition induces its expression. Deletion of

WOR1 did not affect *PTH2* expression, and deletion of *PTH2* did not affect *WOR1* expression, so we constructed a *wor1Δ/Δpth2Δ/Δ* double deletion mutant, and found that deletion of *WOR1* and *PTH2* had no effect on expression of *WOR2* or *CZF1* (Figure S3D). We have found a condition in which *PTH2* is expressed, but it remains to be determined what role Pth2 plays during co-culture.

C. albicans expresses genes associated with hypoxia during suspension co-culture with *C. perfringens*

We used microarrays to measure gene expression in *C. albicans* grown in ambient oxic suspension culture, with and without *C. perfringens* (Figure S4A and Dataset 3). Very few genes were differentially regulated during planktonic co-culture with *C. perfringens*. Using a four-fold cutoff, only two genes were upregulated: *ORF19.2800* and *ORF19.4763*, both of which encode proteins of unknown functions. Using a two-fold cutoff, ten genes were upregulated and nine genes were downregulated. The majority of the upregulated genes are uncharacterized and the downregulated genes span a variety of functions, including the genes encoding an alternative oxidase, Aox2, a surface antigen, Csa1, and genes encoding for proteins involved in sugar acquisition, Hgt1 and Gal7. Interestingly, the gene set differentially regulated in suspension co-cultures is significantly enriched for genes regulated during hypoxic conditions ($p < 1.5 \times 10^{-5}$) [S2], indicating hypoxia may occur during aggregation, which could explain the increased survival of *C. perfringens* when grown aerobically with *C. albicans*.

Supplemental Tables

Table S1, Related to Figure 4. Induction of *C. albicans* aggregation by bacteria during suspension co-culture in oxic or anoxic conditions

| Strain | Oxic, 4 hours | Anoxic, 4 hours | Oxic, 24 hours | Anoxic, 24 hours |
|--|---------------|-----------------|----------------|------------------|
| <i>C. albicans</i> alone | cloudy | cloudy | cloudy | cloudy |
| <i>C. perfringens</i> alone | clear | cloudy | clear | cloudy |
| <i>B. fragilis</i> alone | clear | clear | clear | cloudy |
| <i>E. coli</i> alone | cloudy | cloudy | cloudy | cloudy |
| <i>E. faecalis</i> alone | cloudy | cloudy | cloudy | cloudy |
| <i>K. pneumoniae</i> alone | cloudy | cloudy | cloudy | cloudy |
| <i>C. albicans</i> + <i>C. perfringens</i> | clumps | cloudy | cloudy/clumps | cloudy |
| <i>C. albicans</i> + <i>B. fragilis</i> | cloudy/clumps | cloudy | cloudy | cloudy |
| <i>C. albicans</i> + <i>E. coli</i> | cloudy/clumps | cloudy | cloudy/clumps | cloudy |
| <i>C. albicans</i> + <i>E. faecalis</i> | cloudy/clumps | cloudy | cloudy/clumps | cloudy |
| <i>C. albicans</i> + <i>K. pneumoniae</i> | cloudy | cloudy | cloudy | cloudy |

Table S2, Related to Figure 4. Screen of *C. albicans* deletion mutant strains for aggregation during oxic suspension co-culture with *C. perfringens*. (See spreadsheet for Table S2).

Table S3, Related to Experimental Procedures. Strains. (See spreadsheet for Table S3) [S3–S13].

Table S4, Related to Experimental Procedures. Primers. (See spreadsheet for Table S4).

Supplemental Experimental Procedures

Culture growth and maintenance

C. albicans was streaked from a glycerol stock onto Yeast Peptone Dextrose (YPD) agar plates and grown at room temperature or 30°C. Suspension cultures were grown in YPD media at 30°C, with aeration. *C. perfringens* and *B. fragilis* glycerol stocks were streaked on blood agar plates and grown at 37°C, in an anaerobic jar. Suspension cultures were grown statically in Brain Heart Infusion (BHI) medium, supplemented with 5% fetal bovine serum (BHI-FBS), at 37°C, in an anaerobic jar. *K. pneumoniae*, *E. faecalis*, and *E. coli* glycerol stocks were streaked on Luria Broth (LB) agar plates and grown at 37°C in ambient air. Suspension cultures were grown in BHI-FBS, shaking, at 37°C.

Extended biofilm assay

A 6-well polystyrene plate was incubated overnight with 4 ml bovine serum per well. Bovine serum was removed, wells were washed with 4 ml PBS, and 4 ml of BHI-FBS medium was added. Media was buffered with 67 mM K_2HPO_4 and 33 mM KH_2PO_4 for *K. pneumoniae*, *E. faecalis* and *E. coli* cultures to prevent changes in pH. *C. perfringens* and *B. fragilis* do not change the pH of the culture and do not grow well in the buffered media, so the buffer was omitted for these species. *C. albicans* monocultures were buffered or unbuffered to match co-cultures with each species. Overnight cultures of *C. albicans* and/or bacterial cells were each inoculated to 1×10^7 cfu/ml. Cells were adhered at 37°C, 200 rpm in an ELMI shaker, for 90 min. Unadhered cells were washed off with 4 ml PBS, and fresh 4 ml BHI-FBS was added. Biofilms were allowed to form for 4 or 24 h, at 37°C, 200 rpm in an ELMI shaker, in either oxic or anoxic conditions.

Extended suspension co-cultures

Overnight cultures of each species were inoculated to 1×10^6 cfu/ml in 2ml BHI-FBS media or phosphate buffered BHI-FBS media (see description in Biofilm Assay, above). Cultures were shaken vigorously (225 rpm in an orbital shaker) for 4 h or 24 h at 37°C, either in oxic or anoxic conditions. Cultures were screened visually for aggregation after 4 h of incubation.

Confocal Scanning Laser Microscopy (CSLM)

Biofilms were dyed with 50 µg/ml Concanavalin A – Alexa 594 conjugate (Life Technologies C11253) and 2 µM Syto 13 nucleic acid stain (Life Technologies S7575) for 1 h, 37°C, 200 rpm in an ELMI shaker, in the dark. Medium containing the dye was removed and biofilms were imaged as described previously [S6].

Extended colony forming unit (cfu) assay

C. albicans, and each bacterial species were grown in biofilms or in suspension cultures, alone or pairwise with yeast and bacteria. Cultures were grown under oxic (ambient air) or anoxic (inside an anaerobic jar with a GasPak, BD 260678) conditions. Cells from biofilms were collected at 90 min (immediately after adherence), 4 h, or at 24 h by removing media, washing biofilms with 4 ml PBS, then collecting cells in 4 ml PBS, using a cell scraper and transfer pipet. Cells were vortexed vigorously for 1 min to break up clumps, and serial dilutions were performed. For suspension cultures, serial dilutions were performed directly from cultures. Dilutions were plated onto YPD agar, in ambient air, at 30°C to count *C. albicans* cfu. Dilutions were plated on blood agar, in an anaerobic jar, at 37°C to count *C. perfringens* and *B. fragilis* cfu. Dilutions were plated on LB, in ambient air, at 37°C to count *E. faecalis*, *E. coli*, or *K. pneumoniae* cfu. The initial inoculated media for both biofilms and suspension cultures were also plated to determine the starting cfu/ml. Biofilms were seeded at 1×10^7 cfu/ml and suspension cultures were seeded

at 1×10^6 cfu/ml. At least two biological replicates and three technical replicates were performed for each sample.

Oxygen measurements

Biofilms used for oxygen measurements were grown as described, but on bovine serum coated latex sheets stretched over a ring of polyvinyl chloride (PVC) tubing. We measured cfu/ml to confirm that this substrate produces biofilms capable of supporting *C. perfringens* and *B. fragilis* and found it just as effective as using polystyrene plates as a substrate. We used this set up so that latex could be laid atop an agar plate prior to oxygen measurements, in order to protect the tip of the oxygen sensor probe as it passes from biofilm to substrate. Oxygen concentration was measured as in [S14], but with the following modifications. We used a Unisense STOX-Sensor microelectrode that is based on the Clark-type oxygen sensor, but has two cathodes to amplify the oxygen signal, allowing detection of trace levels of oxygen down to 10 nM [S15]. The sensor electrode was polarized with -0.8 V after connection to a picoampere amplifier on a multimeter. Readings were acquired using SensorTrace pro 3.1.3 software. The calibration curve was a two point curve with a zero point and the fully saturated solution being 271.4 μ M, using the temperature 25°C and a salinity of 0.75%. Atmospheric oxygen was measured in a calibration chamber containing water bubbled with air at 22°C. The zero reading was obtained by measuring a solution of sodium hydroxide and sodium ascorbate at 22°C. To measure biofilms, the sensor probe was positioned above the biofilm, using a Leica MZ 9.5 stereomicroscope to confirm position; readings were taken every 10 μ m, for 3 seconds. The biofilms were covered in PBS during the measurements. At least two technical replicates were performed for each sample. Three successive replicate profiles were taken in each position.

RNA extraction

C. albicans biofilms were grown with or without bacteria, as described above, in 6-well plates for 24 h. Media was removed, biofilms were gently washed with 4 ml PBS, and biofilms were collected in fresh 4 ml PBS by scraping with a cell scraper and transferring to a 50 ml conical tube. 6 wells were pooled from each plate containing one condition. Biofilms samples were centrifuged at 3,000 X g for 5 min and supernatant discarded. Biofilms were stored at -80°C. RNA was extracted as previously described [S16], using the RiboPure – Yeast RNA kit (Ambion, AM1926), following the manufacturer's protocol.

Gene expression microarrays

Gene expression microarrays were performed as previously described [S17]. Briefly, cDNA was synthesized from 8 µg total RNA using Superscript II Reverse Transcriptase (Invitrogen, 18064-014), according to the manufacturer's instruction, and using a mixture of 0.5 mM 3:2 aminoallyl-dUTP (Ambion 8437):dNTPs. cDNA was dye coupled to either Cy3 or Cy5 (GE Healthcare PA23001 or PA25001), then 0.4 µg Cy3-labelled cDNA and 0.4 µg Cy5-labelled cDNA were hybridized onto custom Agilent 8X15k microarrays (AMADID #020166), containing at least two independent probes for each ORF. Slides were scanned on a 4000B Axon Instrument Scanner. Data was analyzed and visualized as described previously [S6] using GenePix Pro software version 7, LOWESS normalization, Cluster version 3.0, and Java TreeView version 1.1.6r2. At least two biological replicates were performed for each condition. We also performed arrays comparing *C. albicans* +*E. coli* K12 type strain to *C. albicans* alone, and found they were very similar to the arrays performed on samples containing the clinical *E. coli* strain used throughout this study (Dataset 1). Gene expression microarray data are reported in Dataset 1 (biofilm co-culture), Dataset 2 (*wor1Δ/Δ* co-culture, additional probes), and Dataset 3 (suspension co-culture). Raw gene expression array data was uploaded to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo; accession #GSE55026)

Strain construction

Deletions strains that were screened in the aggregation assay are listed in Table S2, including the full transcription factor deletion library and several additional mutants published previously [S4]. Table S2 also indicates which strains were confirmed for phenotype by testing a second, independent deletion mutant isolate (8/11 strains) and/or testing a strain complemented with a wild type copy of the deleted gene (9/11 strains). All 11 mutant phenotypes were confirmed with at least one of these methods. *C. albicans* strains with phenotypes in the screen or strains that are used in other assays in this study, as well as bacterial strains, can be found in Table S3. All primer sequences used in this study are listed in Table S4.

Homozygous gene deletion mutant strains and *HIS1* or *LEU2* addback strains were constructed as in [S3], using primers A and B to amplify the 5' flank of the targeted ORF, primers C and D to amplify the 3' flank. Integration was checked using primer E with RZ039 or 41 and primer F with RZ040 or 42 and the ORF was detected with primers G and H. Some strains were signature tagged with a unique 20-mer sequence, using the indicated primers. TF183, was made with pSFS2a-CPH1KO, as described previously [S18]. The *wor1Δ/Δpth2Δ/Δ* strain was constructed by two rounds of *PTH2* gene replacement in the *wor1Δ/Δ* background strain (TF176), using the recyclable SAT1/flipper cassette in plasmid pSFS2A, described previously [S19]. *BRG1* and *RIM101* complementation strains EF93 and EF95 were made by integration of pEF3 and pEF7, respectively, which were derived from pJCP055 [S20]. pEF3 was constructed from pJCP055 using primers PEF143 and 144 to amplify the *BRG1* exon, followed by digestion with XhoI and ligation. Integration was checked with PEF145, ABO3, PEF146, and ABO6. pEF7 was constructed from pJCP055 using primers PEF161 and 162 to amplify the *RIM101* exon, followed by digestion with XhoI and ligation.. Integration was checked with PEF96, ABO3, PEF163, and ABO6.

Light microscopy

15 µl of liquid culture from the suspension co-culture assay was visualized on an Axiovert 200M microscope (Carl Zeiss, Oberkochen, Germany) and images were acquired with Zeiss Axiovision Software, version 4. At least three technical replicates and two biological replicates were examined for each sample.

qPCR

Quantitative real time PCR was performed as described [S21]. Briefly, cDNA was synthesized from 10 µg total RNA using Superscript II Reverse Transcriptase (Invitrogen, 18064-014), according to the manufacturer's instructions. cDNA was amplified in a Bio-rad C1000 Thermocycler and monitored using Sybr Green dye (Sigma 129K2163). Fluorescence was measured on a Bio-rad CFX96 Real-Time System. Data were analyzed using Bio-rad CFX Manager software version 2.0. At least 2 biological replicates were performed for each condition. Gene expression was normalized against either *DYN1* or *PAT1* expression.

Quantification of aggregation

To quantify aggregation in suspension co-cultures, 1 ml of culture was centrifuged for 30 seconds at 500 rpm. 100 µl supernatant and 100 µl pellet were removed, and OD₆₀₀ was read using the Tecan 19 Infinite M1000 PRO microplate reader. At least two biological replicates and two technical replicates were measured for each strain.

Supplemental References

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