

SUPPORTING INFORMATION

Supporting Experimental Procedures

In vitro red pigment formation with arginine and analysis.

Reactions were initially set up by mixing PMS (5 mM) with 50 mM of L-arginine free base (Sigma, A8094) in distilled water yielding a solution with a final pH of 10. At this pH, spontaneous red color formation develops rapidly during 5 minutes of intermittent vortexing at room temperature. Absorption spectra of a 1:25 dilution of the in vitro produced red compound (PMS-arginine) was analyzed by using 1-cm-path-length quartz cuvette in a SpectraMax M5 (Molecular Devices) spectrophotometer. PMS-arginine redox properties were evaluated by reading its absorption spectra after reduction by addition of few crystals of sodium hydrosulfite ($\text{Na}_2\text{S}_2\text{O}_4$) and then oxidation by vortexing for 2 minutes. To react PMS with amino acids at physiological pH (pH 7.5), 25 μl of PMS (100 mM) was mixed with 166.7 μl of HEPES (1.5 M), 5 μl of sodium hydrosulfite (1 M) and 278.3 μl of distilled water prior to the addition of 25 μl of the required amino acid (L-arginine, L-lysine, L-serine or L-alanine; 1M stock solutions). The pH was checked to ensure it remained at 7.5. At neutral pH the reaction proceeds much slower and was allowed to proceed for 24 h in order to evidence red pigmentation. For mass spectrometry analysis, a control reaction lacking $\text{Na}_2\text{S}_2\text{O}_4$ was set up simultaneously. The tubes were centrifuged (14000 rpm/ 2 min) and 1 μl of the crude mixture was diluted in 49 μl of electrospray mass spec solution (MeOH: H_2O : glacial acetic acid - 9:1: 0.1). This solution was then analyzed by LC-MS / ES ionization in positive mode. The crude reaction mixture peak was subtracted from the peak of a mixture consisting of HEPES, $\text{Na}_2\text{S}_2\text{O}_4$ and the amino acid in order to detect any adduct formation. In

the case of PMS reactions in sodium hydrosulfite, mass corresponding to an adduct of PMS with the amino acid was found $[M + H]^+$. For reactions lacking sodium hydrosulfite at pH 7.5, no adduct mass was detected and unreacted PMS mass was observed (See Results section).

Characterization of this red PMS-arginine derivative determined that it exhibited many of the spectral, solubility and redox characteristics previously reported for the coculture red pigment (Gibson *et al.*, 2009), for amino-PMS (Kehrmann and Hanvas, 1913) and for aeruginosin, a red water soluble amine derivative of 5MPCA produced in late monocultures of *P. aeruginosa* (Hansford *et al.*, 1972; Herbert and Holliman, 1969; Holliman, 1969; Katritzky, 1985). These characteristics include a visible spectrum with a band at 520 nm (Fig. 3C), that was reversibly reduced and oxidized. In addition, all compounds remain in the aqueous phase after chloroform extraction, and retained their red spectrum upon acidification. Since amino-PMS and aeruginosin are both derivatives of the 5-methyl phenazinium ring (Fig. S1) with an amine group substituted on the same carbon atom on the fused benzene ring, we conclude that the most likely chemical structure for PMS-arginine and PMS-lysine is that shown in Fig. 3A-*inset*. (See Katritzky reference for a theoretical explanation of the susceptibility of this carbon atom of the phenazinium to nucleophilic attack by amines, to give covalently substituted adducts which are colorless, but are readily oxidized in air to yield the red derivative (Katritzky, 1985)). We have found that these red oxidized derivatives can be reversibly reduced to the colorless derivative. Although we suggest that these proposed chemical structures (Fig. 3A) are the most prominent and most likely, further chemical studies to purify and characterize the products formed with PMS is reacted under these chemical conditions will be needed to confirm our interpretations. A small amount of a product, 2-keto-N-methyl-phenazine (MW 210) formed by substitution of

a hydroxide ion rather than arginine (McIlwain, 1937; Rajagopalan and Handler 1962; Katritsky, 1985, Zaugg, 1964) is apparently present in the mass spectrum of the reaction mixture shown in Fig 3A. Previous chemical studies have revealed that PMS is a reactive molecule and can form a variety of products under basic conditions (McIlwain, 1937).

Reaction of PMS with albumin and analysis.

200 μ l PMS (6 mg/ml water) were reduced to form the emerald green color characteristic of the semiquinoid by addition of a few crystals of $\text{Na}_2\text{S}_2\text{O}_4$ and then added to 600 μ l of a concentrated solution of bovine serum albumin (BSA, 200 mg/ml water, adjusted to pH 8.5 with NaOH) and shaken in air for 60 min. After 18 hours at room temperature, a deep wine red color develops, which persists after a thirty fold dilution in water. The PMS-modified BSA red product has many of the characteristics of the red PMS-arginine derivative described above: it has a visible absorption spectrum with a peak at 520 nm that disappears after adding reductant ($\text{Na}_2\text{S}_2\text{O}_4$), it remains in the aqueous phase upon mixing with chloroform and it retains the red color after acidification. No red product is formed if $\text{Na}_2\text{S}_2\text{O}_4$ is omitted from the reaction mixture unless very strong base is added. Omission of BSA from the reaction mixture at pH 8.5 yields a less intense red color, which after subsequent acidification changes to a yellow /orange pigmentation, indicating the presence of a different product, 2-keto-N-methyl phenazine (McIlwain, 1937; Rajagopalan and Handler, 1962). Similarly to the *C. albicans* pigmented proteins, the red color of the PMS-modified BSA remains with the protein fraction upon precipitation with TCA even if pretreated with 8 M urea. The requirement for $\text{Na}_2\text{S}_2\text{O}_4$ in the reaction mixture at pH 8.5 may reflect both its reducing

power and other characteristics, such as its ability to scavenge dissolved oxygen and thereby stabilize the PMS-semiquinoid at this pH (Zaugg, 1964).

Analyses of PMS and 5MPCA induced intracellular fluorescence by FACS.

For analysis of the effects of cellular fluorescence, *C. albicans* was spot inoculated (10 μ l) from exponential phase cultures onto YNBA medium with or without (control) 1 mM PMS for 24 h. Approximately, 15 colonies were suspended in 1 ml of saline solution, washed by centrifugation with saline solution twice and then diluted to an absorbance of 3.0. That suspension was divided into 6 tubes and either used as controls or treated with 100 mM of $\text{Na}_2\text{S}_2\text{O}_4$. For analysis of killed cells, suspensions were heated for 30 min at 80°C. CFUs were determined before and after treatment by plating serial dilutions of each suspension as we described previously (see the Experimental Procedures section of the paper). No change in the number of CFUs was found after treatment with $\text{Na}_2\text{S}_2\text{O}_4$; however, after heating, fungal cells did not form colonies even after prolonged incubation times. Flow cytometry analyses of each cell suspension were performed as was previously described in the paper.

Table S1. Bacterial and fungal strains used in this study

Strain	Description	DH # a	Source or reference
<i>Pseudomonas aeruginosa</i> strains			
PA14 WT	WT	123	(1)
PA14 <i>phzM::TnM</i>	TnM mutant, 5MPCA negative	693	(2)
PA14 <i>phzS::TnM</i>	TnM mutant, PYO negative	698	(2)
<i>Candida albicans</i> strains			
SC5314	WT	35	(3)
CAI-4	SC5314 derivative, Uri – <i>ura3::imm434/ura3::imm434</i>	332	(4)
CU2	CAI-4 derivative, Uri+ reference for 1F541	1209	(5)
1F541 <i>cat1/cat1 (cta1/cta1)</i>	<i>ura3/ura3, /cat1::hisG/cat1::hisG</i> . Catalase deficient strain	1214	(5)
pGFP	CAI-4 derivative, Uri+. <i>ADHI-GFP</i> control fusion inserted in the <i>RSP1</i> locus	937	(6)
CAT1 (Catalase)	CAI-4 derivative, Uri+. <i>CAT1-GFP</i> reporter fusion inserted in the <i>RSP1</i> locus	939	(7)
TTR1 (Thioredoxin system)	CAI-4 derivative, Uri+. <i>TTR1-GFP</i> reporter fusion from (7) inserted in the <i>RSP1</i> locus	1569	This study
TRX1 (Thioredoxin system)	CAI-4 derivative, Uri+. <i>TRX1-GFP</i> reporter fusion from (7) inserted in the <i>RSP1</i> locus	1570	This study

^a Lab collection number for reference.

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3. Gillum A.M., Tsay E.Y., Kirsch D.R. (1984) Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae ura3* and *E. coli pyrF* mutations. *Mol Gen Genet* 198(1):179-82.

4. Leberer E., et al. (2001) Ras links cellular morphogenesis to virulence by regulation of the MAP kinase and cAMP signalling pathways in the pathogenic fungus *Candida albicans*. *Mol Microbiol* 42:673-87.
5. Nakagawa Y. (2008) Catalase gene disruptant of the human pathogenic yeast *Candida albicans* is defective in hyphal growth, and a catalase-specific inhibitor can suppress hyphal growth of wild-type cells. *Microbiol Immunol* 52(1):16-24.
6. Barelle, C.J., Manson, C.L., MacCallum, D.M., Odds, F.C., Gow, N.A., and Brown, A.J. (2004) GFP as a quantitative reporter of gene regulation in *Candida albicans*. *Yeast* 21: 333-340.
7. Enjalbert, B., MacCallum, D.M., Odds, F.C., and Brown, A.J. (2007) Niche-specific activation of the oxidative stress response by the pathogenic fungus *Candida albicans*. *Infect Immun* 75: 2143-2151.

Supporting Figures

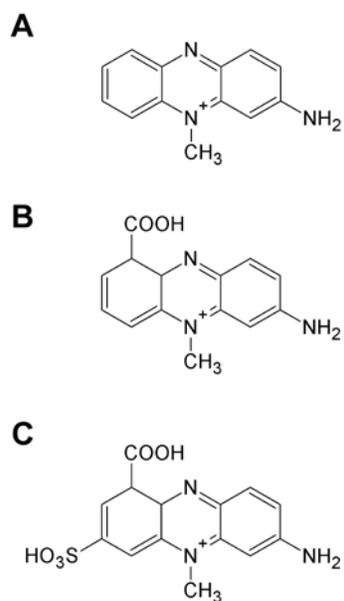


Fig. S1. Chemical structures of methylphenazinium derivatives. A. 2-amino-10-methylphenazinium methyl sulfate (amino-PMS). B. Aeruginosin A. C. Aeruginosin B.

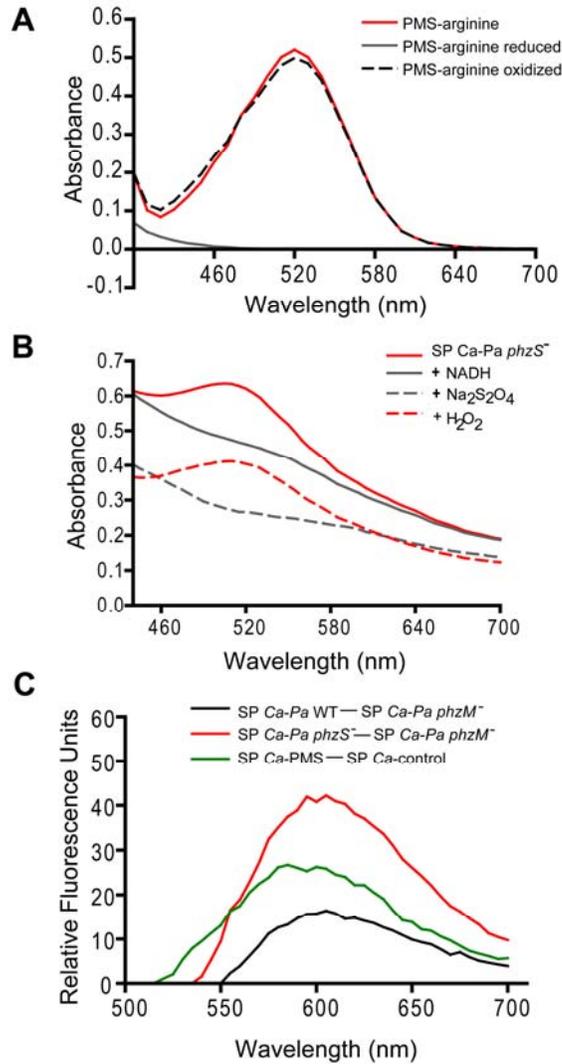


Fig. S2. Red-pigmented derivatives spectra characteristics. A. Absorption spectra of the in vitro-produced red compound formed by reacting PMS with arginine (PMS-arginine). The same pigment was reduced with a few crystals of Na₂S₂O₄ (PMS-arginine reduced) and then reoxidized (PMS-arginine oxidized) by vortexing in air for 2 minutes. B. Resulting absorption spectra of *C. albicans* soluble proteins (SP) from co-cultures with *P. aeruginosa phzS::TnM* (*Ca-Pa phzS*⁻) after reduction by addition of either 2 mM NADH or 80 mM Na₂S₂O₄, and reoxidation by treatment with 280 mM H₂O₂. C. Fluorescence emission spectra of *C. albicans*

SP extracted from cultures with *P. aeruginosa* WT (*Ca-Pa* WT), *phzS*::TnM (*Ca-Pa phzS*⁻) or 1 mM PMS (*Ca-PMS*). To minimize the effects of non-specific fluorescence in the turbid extracts, these were obtained by subtracting (-) the background fluorescence of SP of *C. albicans* cultured with *phzM*::TnM (*Ca-Pa phzM*) or without PMS (*Ca-control*). The non-pigmented SP only showed non-specific fluorescence below 550 nm, while the red pigmented SP exhibited maximal fluorescence near 600 nm. Excitation was at 488 nm, with a cutoff at 515 nm, using a SpectraMax M2 spectrophotometer.

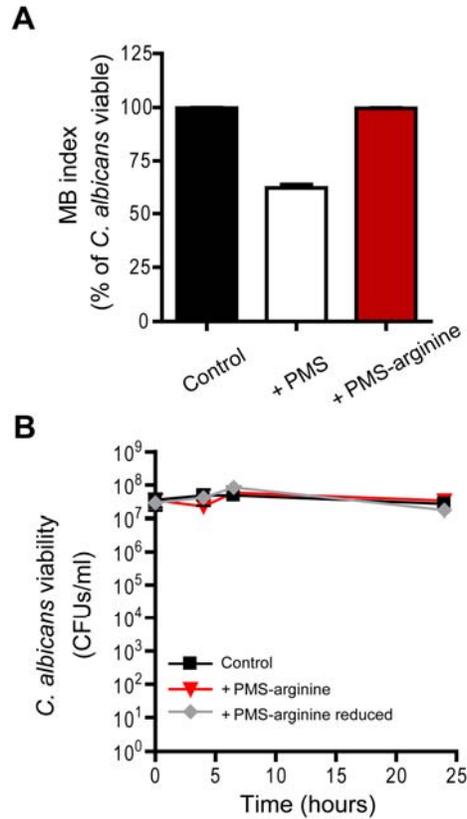


Fig. S3. Analysis of toxicity of in vitro-synthesized PMS-arginine pigment during growth and in cell suspensions. A. *C. albicans* from exponential phase cultures grown to an absorbance (OD_{600}) of 3.0 in 5 ml YPD were spot inoculated (10 μ l) onto plates of YNBA with or without 1 mM of PMS or PMS-arginine and then grown for 72 h. The concentration of the PMS-arginine reaction product was estimated assuming that all 5 mM PMS was covalently bound to arginine in the chemical reaction described in the Experimental Procedures section, since no free unreacted PMS was found after mass spectrometry analysis. The viability of yeast cells sampled from colonies-grown for 72 h, was assessed by MB staining as described in the Experimental Procedure section (n=2). B. *C. albicans* ($\sim 10^7$ cells) was suspended in 500 μ l of 100 mM HEPES buffer pH 7.5 (control) containing ~ 1 mM PMS covalently modified by arginine either in its oxidized form (PMS-arginine) or previously reduced by

adding 100 mM of $\text{Na}_2\text{S}_2\text{O}_4$ (PMS-arginine reduced). *C. albicans* survival for each treatment was determined by CFU counts on YNBA medium at the beginning of the assay and after the specified time points (n=2). *C. albicans* viability was not affected by $\text{Na}_2\text{S}_2\text{O}_4$ itself (data not shown). The addition of $\text{Na}_2\text{S}_2\text{O}_4$ to the PMS-arginine derivative yields initially a colorless solution. Reduction was not possible by adding directly ascorbic acid (AA) into the red PMS-arginine containing buffer.

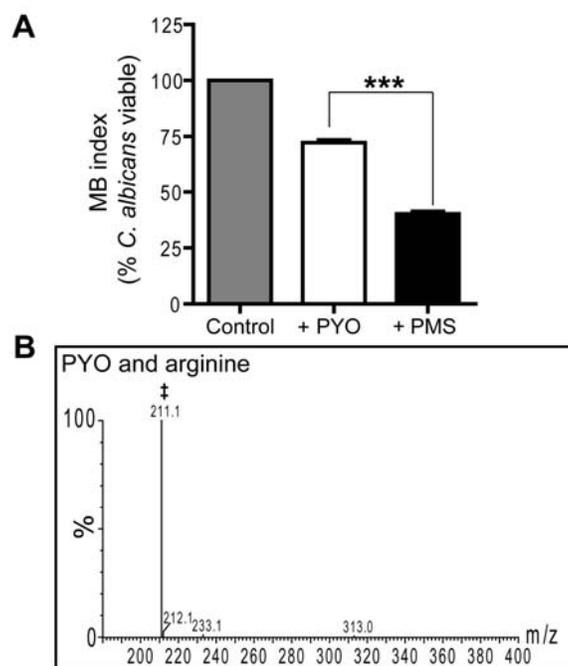


Fig. S4. Comparison of PYO and PMS effects on *C. albicans*. A. Yeast cells were grown for 72 h at 30°C on YNBA medium with or without (control) 1 mM of PYO or of PMS. *C. albicans* survival was measured by MB staining of colonies sampled simultaneously after 72 h (n=3) *** $P < 0.01$. Data are representative of two independent experiments in which all growth conditions were compared in parallel. B. LC-MS/ES ionization analysis of the chemical reaction of PYO with arginine. The conditions were the same as used for the chemical reaction of PMS with arginine and other amino acids, in HEPES buffer pH7.5, with and without $\text{Na}_2\text{S}_2\text{O}_4$ (See Experimental Procedures). † represents the unreacted pyocyanin mass.

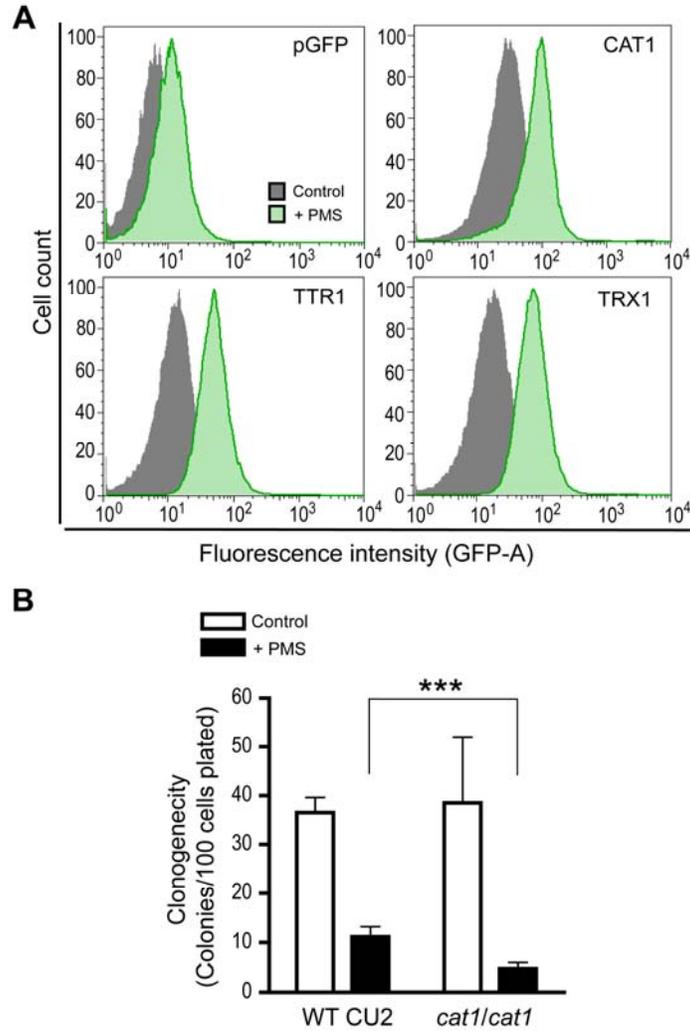


Fig. S5. Oxidative stress and cell death in *C. albicans*. **A.** Expression of ROS responsive genes in *C. albicans* cells exposed to PMS. The strains containing *CAT1*-, *TTR1*- and *TRX*-*GFP* and the pGFP were incubated on YNBA medium in presence or absence (control) of 1 mM PMS at 30°C. Data are from flow cytometry analysis done after 18 hours of treatment. **B.** Clonogenicity of *C. albicans* *CAT1/CAT1* (WT CU2) or *cat1/cat1* grown on YNBA medium alone (control) and with 1 mM PMS for 72 hours. Fungal survival was determined by counting the number of colonies grown (of 100 cells plated) on YPD medium after 3 days of incubation (n=3).

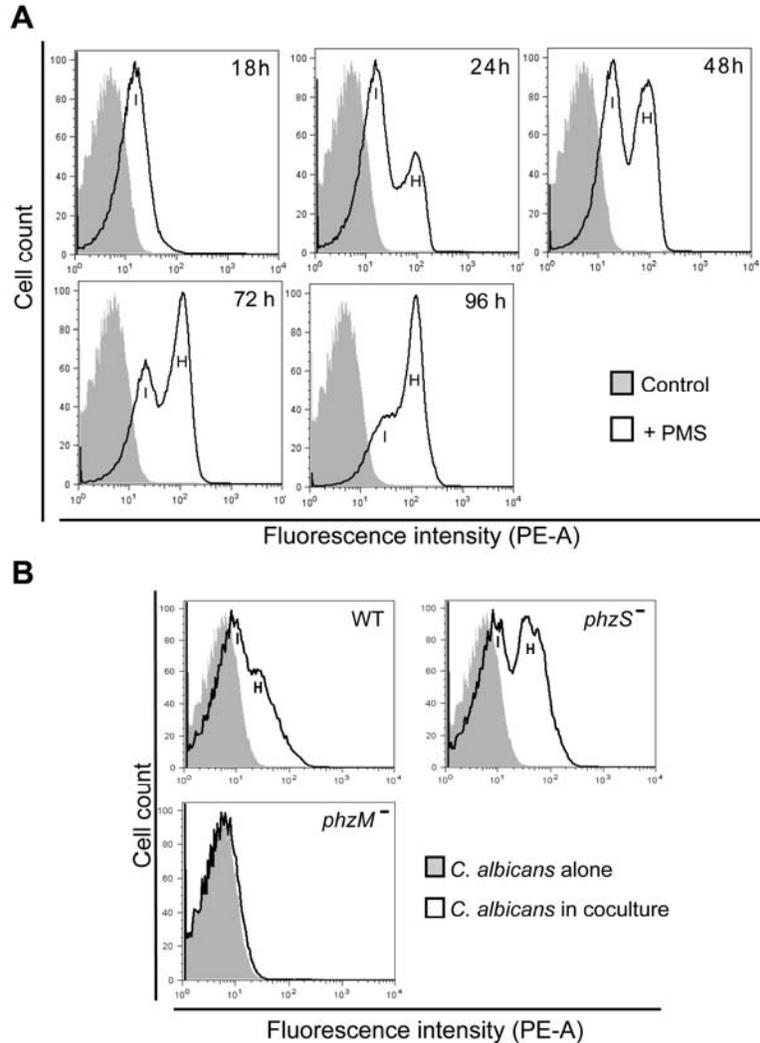


Fig. S6. PMS and 5MPCA induced fluorescence. A. Kinetics of fluorescence development in PMS-exposed *C. albicans* as determined by flow cytometry analysis. Solid histograms indicate background fluorescence in *C. albicans* grown on YNBA medium without PMS. *C. albicans* cells exhibited either intermediate (I) or high (H) fluorescence in the phycoerythrin (PE) channel. Data are representative of two independent experiments. B. Flow cytometry analysis of *C. albicans* cocultured for 24 hours with the indicated *P. aeruginosa* strains also showed I and H levels of intracellular fluorescence. Solid histograms indicate background fluorescence in *C. albicans* grown alone.

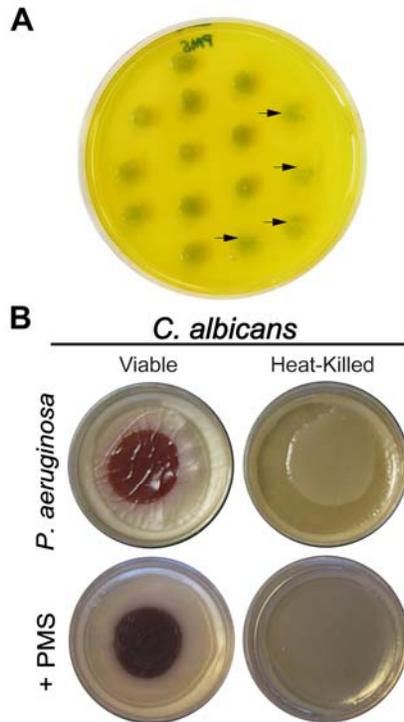


Fig. S7. *C. albicans* colony reducing properties and fungal metabolic activity are required for red pigment development. A. *C. albicans* was spot inoculated (10 μ l) into PMS containing medium. After ~18 h of incubation, the fungal colonies develop a green emerald coloration, which was also observed on the agar surface once the colony was removed (arrow). B. Lawns of *C. albicans*, prepared by growth for 48 h as described in Experimental Procedures, were left untreated (viable) or heat-killed at 60°C for 12 h prior to inoculation with *P. aeruginosa* PA14 or prior to addition of 50 μ l of 20 mM PMS. Pictures were taken 48 h after further incubation at 30°C