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) were 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 ($Na_2S_2O_4$) 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 $Na_2S_2O_4$ 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, $Na_2S_2O_4$ and the amino acid in order to detect adduct formation. In the case of PMS reactions

in sodium hydrosulfite, mass corresponding to an adduct of PMS with the amino acid was found $[M + H]^+$. As described in the results section, in reactions lacking sodium hydrosulfite at pH 7.5, only the mass corresponding to unreacted PMS was observed.

Characterization of this red PMS-arginine derivative determined that it exhibited many of the spectral, solubility and redox characteristics previously reported for the co-culture 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, 1985) 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 readily oxidized in air to yield a red derivative). 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 when 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; Rajagopolan 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 (Mclwain, 1937).

Reaction of PMS with albumin and analysis.

200 μl PMS (6 mg/ml water) were reduced to form the emerald green color characteristic of the semiquinoid by addition of a few crystals of $Na_2S_2O_4$. This was added to 600 μl of a concentrated solution of bovine serum albumin (BSA, 200 mg/ml water, adjusted to pH 8.5 with NaOH) and then shaken in air for 60 min. After 18 hours at room temperature, a deep wine red color developed, which persisted after a thirty fold dilution in water. The PMS-modified BSA red product had many of the characteristics of the red PMS-arginine derivative described above: it had a visible absorption spectrum with a peak at 520 nm that disappeared after adding reductant ($Na_2S_2O_4$), it remained in the aqueous phase upon mixing with chloroform and it retained the red color after acidification. No red product was formed if $Na_2S_2O_4$ was omitted from the reaction mixture unless very strong base was added. Omission of BSA from the reaction mixture at pH 8.5 yielded a less intense red color, which after subsequent acidification changed to a yellow /orange pigmentation, indicating the presence of a different product, 2-keto-N-methyl phenazine (Mclwain, 1937; Rajagopalan and Handler, 1962). Similar to the *C. albicans* pigmented proteins, the red color of the PMS-modified BSA remained with the protein fraction upon precipitation with TCA even if pretreated with 8 M urea. The requirement for $Na_2S_2O_4$ 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 μl) 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 $Na_2S_2O_4$. For analysis of killed cells, suspensions were heated for 30 min at 80^{o}C. CFUs were determined before and after treatment by plating serial dilutions of each suspension as we described previously (see Experimental Procedures). No change in the number of CFUs was found after treatment with $Na_2S_2O_4$; 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
Pseudomonas aeruginosa strains			
PA14 WT	WT	123	(1)
PA14 *phzM*::Tn*M*	Tn*M* mutant, 5MPCA negative	693	(2)
PA14 *phzS*::Tn*M*	Tn*M* mutant, PYO negative	698	(2)
Candida albicans strains			
SC5314	WT	35	(3)
CAI-4	SC5314 derivative, Uri – *ura3::imm434/ura3:: imm434*	332	(4)
CU2	CAI-4 derivative, Uri+ reference for 1F541	1209	(5)
1F541 *cat1*/*cat1* (*cta1*/*cta1*)	*ura3*/*ura3*, *cat1*::*hisG*/c*at1*::*hisG* Catalase deficient strain	1214	(5)
cap1/cap1	*ura3*/*ura3*, *CAT1/CAT1*, *cap1*::*hisG*/c*ap1*::*hisG* CAP1 deficient strain	1216	(5)
pGFP	CAI-4 derivative, Uri+. *ADH1-GFP* control fusion inserted in the *RSP1* locus	937	(6)
CAT1-GFP	Catalase promoter fusion strain. CAI-4 derivative, Uri+. *CAT1-GFP* reporter fusion inserted in the *RSP1* locus.	939	(7)
TTR1-GFP	Thioredoxin system promoter fusion strain. CAI-4 derivative, Uri+. *TTR1-GFP* reporter fusion from (7) inserted in the *RSP1* locus	1569	This study
TRX1-GFP	Thioredoxin system promoter fusion strain. CAI-4 derivative, Uri+. *TRX1-GFP* reporter fusion from (7) inserted in the *RSP1* locus	1570	This study
RM100	WT, Uri + *ura3Δ::im434/ura3Δ::imm434 hisIΔ::hisG/hisIΔ::hisG-URA3-hisG*	1270	(8)
hog1/hog1	*ura3Δ::im434/ura3Δ::imm434 hisIΔ::hisG/hisIΔ::hisG hog1::hisG-URA3-hisG* HOG1 MAPK deficient strain	1269	(9)

[a] Lab collection number for reference.

1. Rahme L.G*., et al.* (1995) Common virulence factors for bacterial pathogenicity in plants and animals. *Science* 268(5219):1899-902.

2. Liberati N.T., et al. (2006) An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. Proc Natl Acad Sci U S A 103(8):2833-8.

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.

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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.

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8. Fonzi, W.A., and Irwin, M.Y. (1993) Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* 134: 717-728.

9. San José, C., Alfonso-Monge, R., Pérez-Díaz, R. M., Pla, J. and Nombela, C. (1996) The mitogen-activated protein kinase homolog *HOG1* gene controls glycerol accumulation in the pathogenic fungus *Candida albicans*. *J Bacteriol* 178: 5850-5852.

Supporting Figures

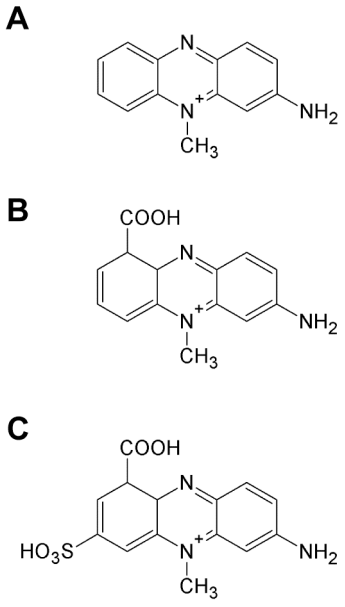

Fig. S1. Chemical structures of methylphenazinium derivatives. A. 2 amino-10-methyl phenazinium methyl sulfate (amino-PMS). (Only the phenazinium ion is shown). B. Aeruginosin A. C. Aeruginosin B.

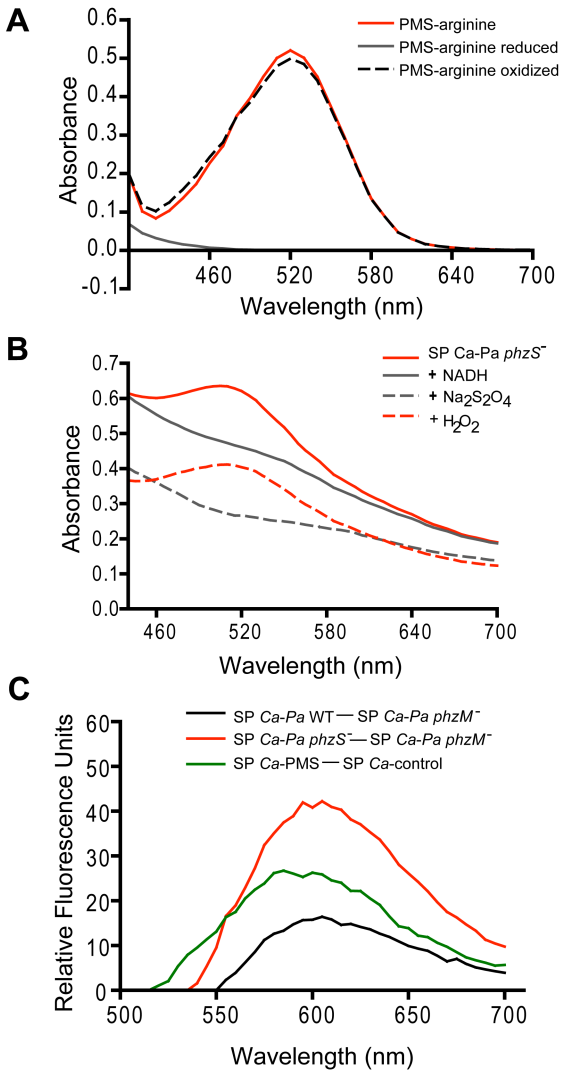

Fig. S2. Red-pigmented derivatives spectra characteristics. A. Absorption spectra of the in vitro-produced red compound formed by reacting PMS with arginine (PMS-arginine). The same pigment was reduced with a few crystals of $Na_2S_2O_4$ (PMS-arginine reduced) and then reoxidized (PMS-arginine oxidized) by vortexing in air for 2 minutes. B. Resulting absorption spectra of *C. albicans* soluble proteins (SP) from co-cultures with *P. aeruginosa phzS*::Tn*M* (*Ca-Pa phzS*$^-$) after reduction by addition of either 2 mM NADH or 80 mM $Na_2S_2O_4$, and reoxidation by treatment with 280 mM H_2O_2. C. Fluorescence emission spectra of *C. albicans*

SP extracted from cultures with *P. aeruginosa* WT (*Ca*-*Pa* WT), *phzS*::Tn*M* (*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*::Tn*M* (*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.

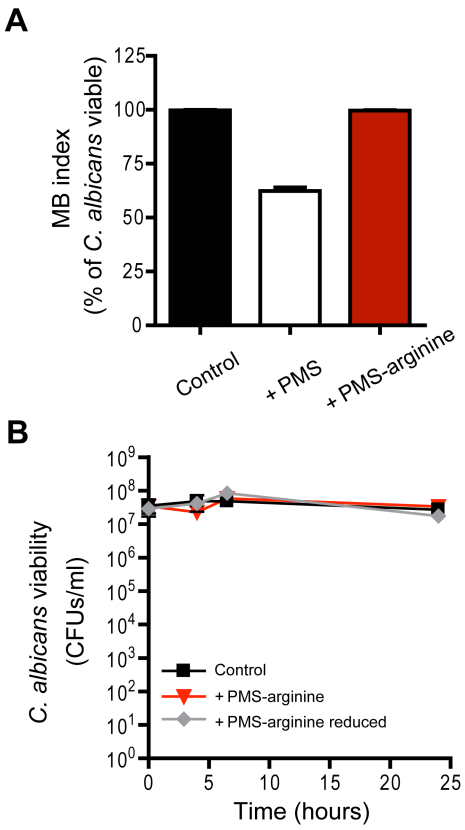

Fig. S3. Analysis of toxicity of in vitro-synthesized PMS-arginine pigment during growth and in cell suspensions. A. *C. albicans* from exponential phase cultures grown to an absorbance (OD_{600}) of 3.0 in 5 ml YPD were spot inoculated (10 µl) onto plates of YNBA with or without 1 mM of PMS or PMS-arginine 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* (~10^7 cells) was suspended in 500 µl 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 $Na_2S_2O_4$ (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 $Na_2S_2O_4$ itself (data not shown). The addition of $Na_2S_2O_4$ 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.

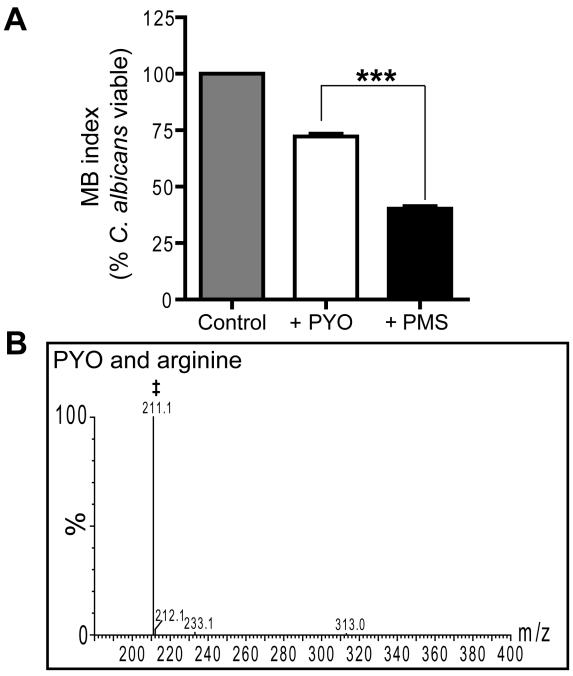

Fig. S4. Comparison of PYO and PMS effects on *C. albicans*. A. Yeast cells were grown for 72 h at 30°C on YNBA medium with or without (control) 1 mM of PYO or of PMS. *C. albicans* survival was measured by MB staining of colonies sampled simultaneously after 72 h (n=3) ***$P<0.01$. Data are representative 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 $Na_2S_2O_4$ (See Experimental Procedures). ‡ represents the unreacted pyocyanin mass.

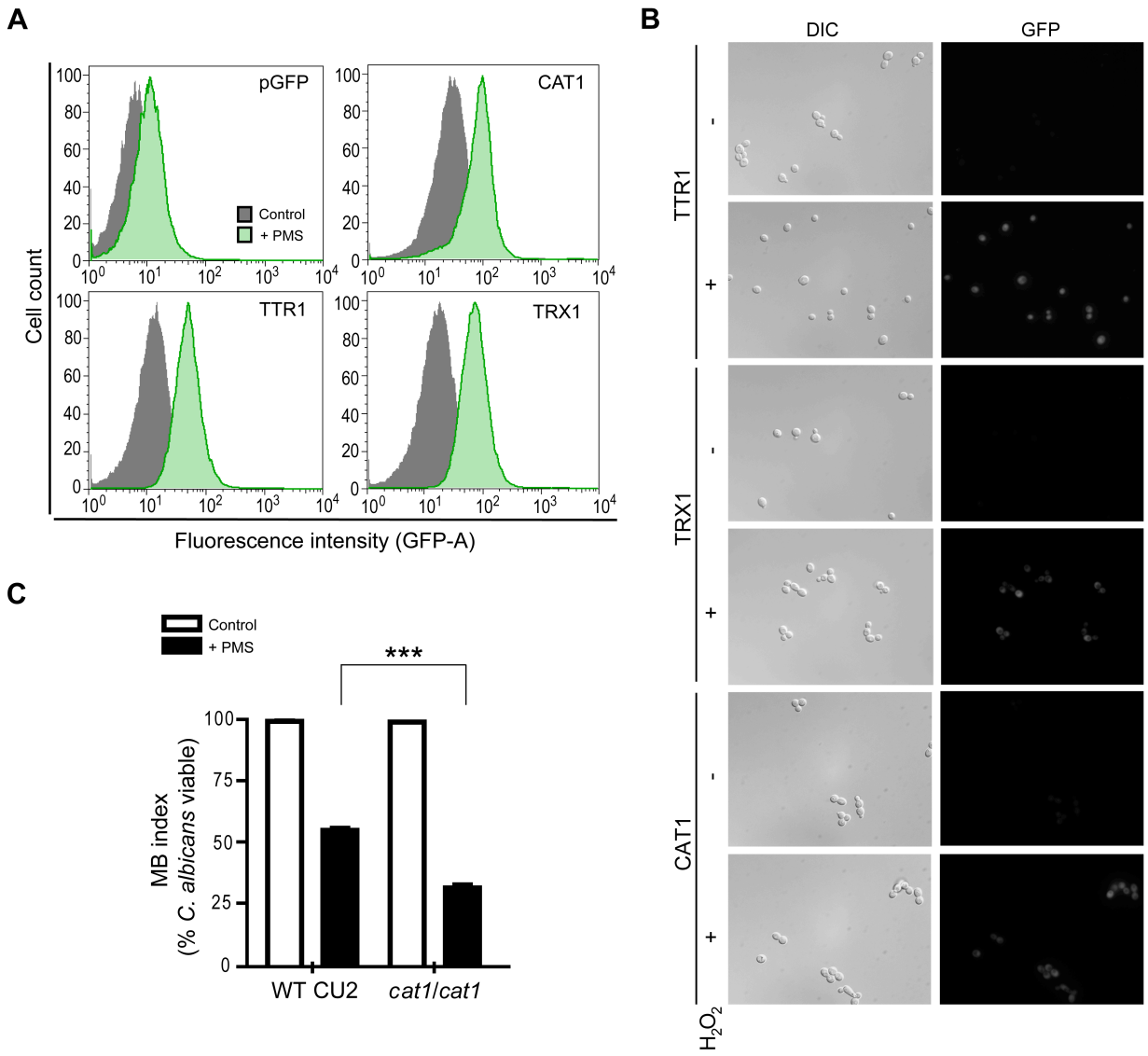

Fig. S5. Oxidative stress and cell death in *C. albicans*. A. Expression of ROS-responsive genes in *C. albicans* cells exposed to PMS. The strains containing *CAT1*-, *TTR1*- and *TRX-GFP* and the pGFP were incubated on YNBA medium in presence or absence (control) of 1 mM PMS at 30°C. Data are from flow cytometry analysis done after 18 hours of treatment. B. Microscopic view of epifluorescence (GFP) and DIC images of a suspension of *C. albicans TTR1*, *TRX1* and *CAT1* promoter fusion strains after exposure to 10 mM H_2O_2 for 2 hours compared to controls. C. *C. albicans CAT1/CAT1* (WT CU2) or *cat1/cat1* were grown on YNBA medium alone

(control) or with PMS for 72 hours. Fungal survival determined by MB staining (n=3). Data are representative of at least two independent experiments ***$P<0.01$.

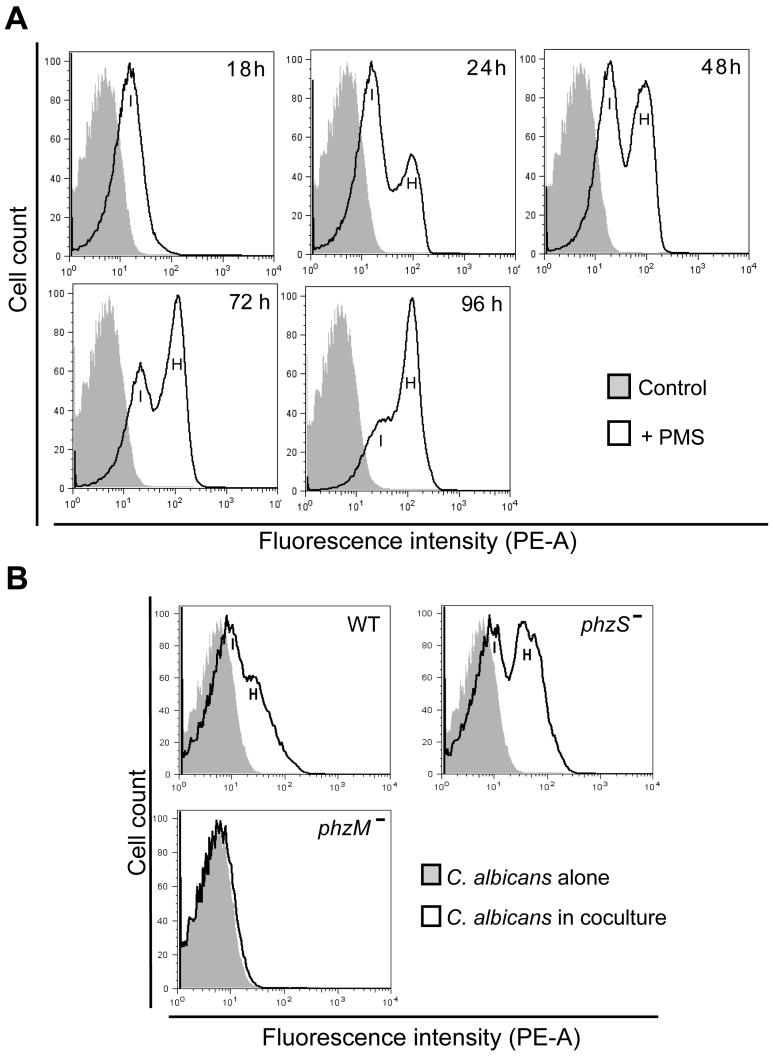

Fig. S6. PMS and 5MPCA induced fluorescence. A. Kinetics of fluorescence development in PMS-exposed *C. albicans* as determined by flow cytometry analysis. Solid histograms indicate background fluorescence in *C. albicans* grown on YNBA medium without PMS. *C. albicans* cells exhibited either intermediate (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.

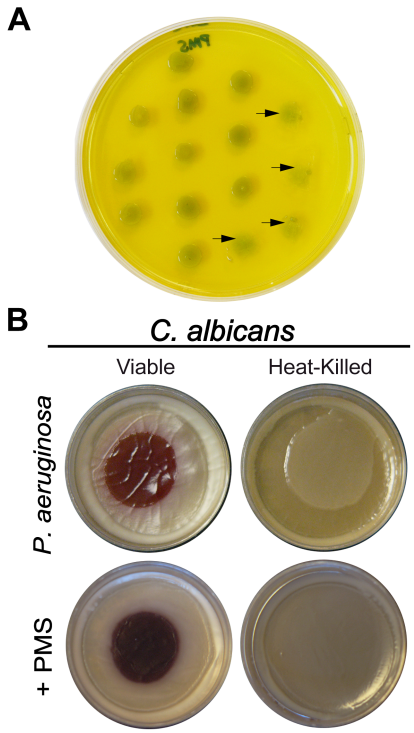

Fig. S7. *C. albicans* colony reducing properties and fungal metabolic activity are required for red pigment development. A. *C. albicans* was spot inoculated (10 μl) into PMS containing medium. After ~18 h of incubation, the fungal colonies develop a green emerald coloration, which was also observed 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^{o}C for 12 h prior to inoculation with *P. aeruginosa* PA14 or prior to addition of 50 μl of 20 mM PMS. Pictures were taken 48 h after further incubation at 30^{o}C