

Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*

Ifat Rubin-Bejerano*, Iain Fraser*[†], Paula Grisafi*, and Gerald R. Fink*[‡]

*Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology, Nine Cambridge Center, Cambridge, MA 02142; and [†]Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114

Contributed by Gerald R. Fink, July 17, 2003

The transcriptional profiles of yeast cells that have been phagocytosed by either human neutrophils or monocytes were compared by using whole genome arrays. After phagocytosis by neutrophils, both *Saccharomyces cerevisiae* and *Candida albicans* respond by inducing genes of the methionine and arginine pathways. Neither of these pathways is induced upon phagocytosis by monocytes. Both fungi show a similar induction of these pathways when transferred from amino acid-rich medium to amino acid-deficient medium. These data suggest that the internal phagosome of the neutrophil is an amino acid-deficient environment.

Ingestion of a microorganism by mammalian cells exposes that organism to a novel environment. Transcriptional profiling of a microorganism with a well-annotated genome sequence offers a unique window into the response of that organism to phagocytosis. Used in this way, the microorganism becomes a functional bioprobe of the phagocyte's microenvironment. For example, upon ingestion by macrophages *Saccharomyces cerevisiae* undergoes a key metabolic shift: the induction of the glyoxylate shunt (1). This pathway is also induced upon ingestion of bacteria (*Mycobacterium tuberculosis*) (2) and other fungi [*Cryptococcus neoformans* (3), *Candida albicans* (1), and *Leptosphaeria maculans* (4)]. This shift in metabolism has been interpreted as a response to the glucose-poor environment of the macrophage, and the ability to make that shift appears to contribute to the virulence of some pathogens.

In this report we use transcriptional profiling of *S. cerevisiae* to analyze the phagosomal microenvironment of the human neutrophil. The yeast transcriptional response is dominated by induction of the methionine and arginine amino acid biosynthetic genes. This transcriptional response could be mimicked *in vitro* by transferring yeast from an amino acid-rich environment to one deficient in amino acids. The response of *Saccharomyces* to neutrophils is distinct from that to monocytes, which do not induce these amino acid pathways. The human pathogen *C. albicans* also induces the transcription of a similar set of amino acid biosynthetic genes upon exposure to human neutrophils. These data suggest that the human neutrophil phagosome is amino acid-deficient, and indicate an amino acid-deprivation response that is conserved between *S. cerevisiae* and *C. albicans*.

Materials and Methods

Isolation of Neutrophils and Monocytes and Human Serum Preparation. Using Histopaque 1077 and Histopaque 1119 (Sigma) per manufacturer's instructions, we isolated neutrophils and monocytes from fresh human blood collected from healthy volunteers in accordance with a protocol approved by the Massachusetts Institute of Technology Committee on Use of Humans as Experimental Subjects. Monocytes were separated from lymphocytes on the basis of their differential adherence to plastic. Human serum was prepared from the same blood donors by using Vacutainer SST gel-containing tubes (Becton Dickinson) and following the manufacturer's protocol. Where indicated, human serum was heat inactivated by incubation at 56°C for 30 min.

Coincubation of Phagocytes with Fungal Cells. Neutrophils or monocytes were mixed with *S. cerevisiae* (*Saccharomyces*) or *C. albicans* (*Candida*) cells at ratio of one phagocyte to five yeast cells. The *Saccharomyces* strains were EM93, S288C, or *gcn4Δ* isogenic with S288C. The *Candida* strain was SC5314. The fungal cells were incubated with neutrophils, monocytes, or alone in RPMI medium 1640 with 10% human serum at 37°C for 10, 20, 40, 60, or 80 min. Yeast viability was assessed by using the XTT tetrazolium dye assay (5). The neutrophils were lysed in water provided with the RNase inhibitor RNasin (Promega), and the yeast released was washed and frozen quickly in phenol.

Microarray Procedure. Total RNA and mRNA were prepared as described (1). For *Saccharomyces* first- and second-strand synthesis, *in vitro* transcription, hybridization, and scanning were done according to the Affymetrix protocol. For *Candida* total RNA was used to amplify mRNA by using RiboAmp (Ambion, Austin TX). Amino-allyl reverse transcription, hydrolysis, coupling, and quenching were done as described at <http://cmgm.stanford.edu/pbrown/protocols/aadUTPCouplingProcedure.htm>. Hybridization, washing, and scanning were done as described at www.whitehead.mit.edu/CMT/MicroarrayProtocols.html.

Microarray Analysis. Microarrays were normalized and floored at 20. Ratios of expression from yeast cultured in an experimental condition divided by that from the yeast control (from the same time point and culture medium) were calculated. Induced and repressed genes were defined as those with an expression ratio greater than two standard deviations from the mean for a given experiment. Only genes that were consistently induced or repressed in two experimental duplicates were considered as induced or repressed. Clustering analysis was performed by using CLUSTER and TREEVIEW (<http://rana.lbl.gov/EisenSoftware.htm>).

Raw data and supplementary information are available at http://jura.wi.mit.edu/fink_public/neutrophils/.

Northern Analysis. The RNA extracted from *Candida* isolated from neutrophils gave evidence of degraded RNA that affected its quantification by absorbance readings. To isolate the high molecular weight RNA, total RNA was absorbed to RNeasy Mini Kit columns (Qiagen, Valencia, CA) that absorb RNA larger than 200 bases. This eluted RNA was quantified, and 15 μg was loaded on a gel. The RNA from control experiments was subject to the same treatment and loaded in serial dilutions on the same gel.

Results

Ingestion by Neutrophils Induces the Methionine and Arginine Pathways. To determine the changes in gene expression upon phagocytosis of *Saccharomyces* by neutrophils, we compared the

[†]To whom correspondence should be addressed. E-mail: gink@wi.mit.edu.

© 2003 by The National Academy of Sciences of the USA

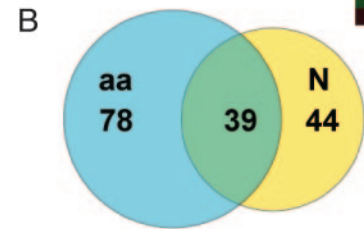
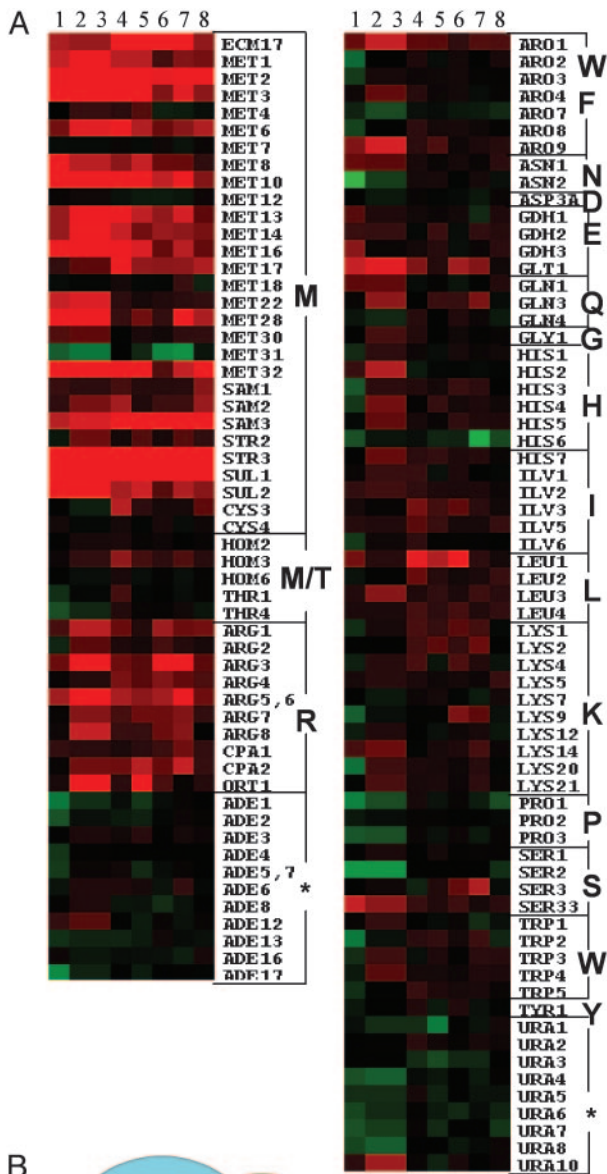


Fig. 2. In *Saccharomyces* the response to ingestion by neutrophils is similar to that engendered by amino acid deprivation. (A) *Saccharomyces* was cultured with neutrophils in RPMI medium 1640 containing 10% fresh autologous human serum (columns 4–8) or in minimal medium without neutrophils (columns 1–3) for 40 min (columns 1–5 and 8) or 60 min (columns 6 and 7). Yeast strains were wild type (columns 2–8) or *gcn4Δ* (column 1). Strain backgrounds were EM93 (columns 3–7) or S288C (columns 1, 2, and 8). For purposes of illustration, amino acid and nucleic acid biosynthetic genes were selected for cluster analysis. *ADE* and *URA* genes served as control genes (*). Experiments were clustered by using CLUSTER and TREEVIEW. (B) The transcriptional profile in response to neutrophils (N) was compared with amino acid (aa) deprivation conditions.

Response to Neutrophils Is Distinct from Exposure to Hydrogen Peroxide or Stress. Neither the methionine nor the arginine pathway was induced by exposure to hydrogen peroxide, a critical component of neutrophil-mediated host defense (8).

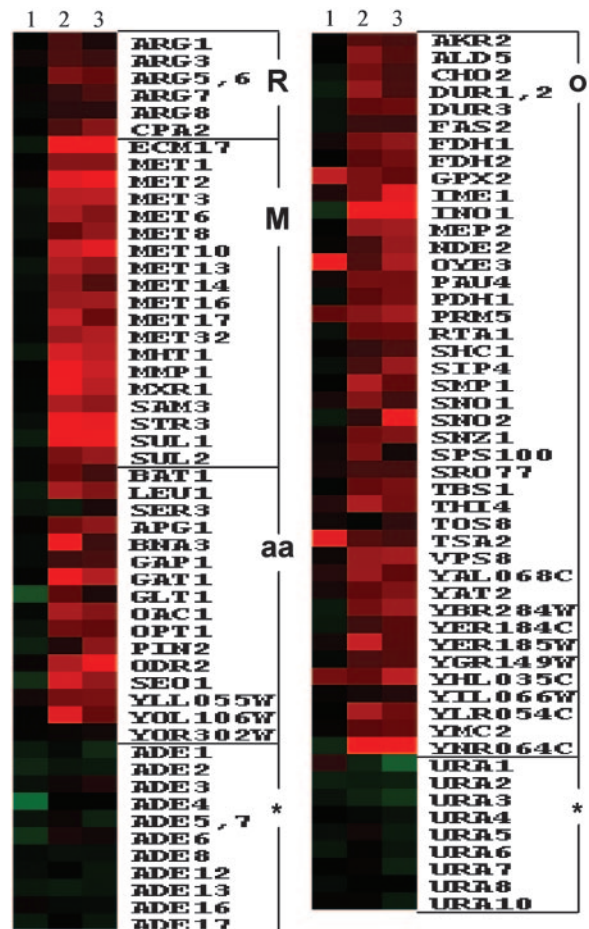


Fig. 3. The expression profile of yeast in response to neutrophils is not dominated by a response to oxidative stress. Yeast cells were cultured in the presence of 0.4 mM H_2O_2 (column 1) or with neutrophils (columns 2 and 3) for 40 min. All 83 genes meeting criteria for induction were selected for illustration purposes. Genes were divided into four groups: methionine (M), arginine (R), other amino acid genes (aa), and other genes (o). *ADE* and *URA* genes served as control (*) genes. Experiments were clustered by using CLUSTER and TREEVIEW.

When *Saccharomyces* was exposed to 0.4 mM hydrogen peroxide for 40 min, 173 genes were induced, of which 7 were also induced by neutrophils: *YHL035C*, *PRM5*, *THI4*, *OYE3*, *YAL068C*, *GPX2*, and *TSA2* (Fig. 3). Moreover, other genes induced by hydrogen peroxide (e.g., *SOD2*, *CCP1*, and *CTAI1*), were not consistently expressed in response to neutrophils.

The transcription profile of yeast inside neutrophils is also distinct from that of each of the published datasets of yeast genes induced in response to a large number of diverse environmental stress conditions (6, 9). Of the 127 genes induced in both datasets in response to different stress conditions, only 1 is also induced by neutrophils.

Neutrophil-Induced Transcription Is Distinct from That Displayed During the Cell Cycle. A number of methionine biosynthetic genes (the “*MET* cluster”) are transiently expressed during S phase of the cell cycle (10), raising the possibility that the induction of methionine genes in response to neutrophils is a consequence of cell cycle arrest. However, there are significant differences between the *MET* cluster cell cycle signature and our data. First, seven genes in the *MET* cluster were not induced by neutrophils: *ICY2*, *MET11*, *YLR302C*, *MET28*, *SER33*, *YNL276C*, and *MUP1*. Second, a number of the

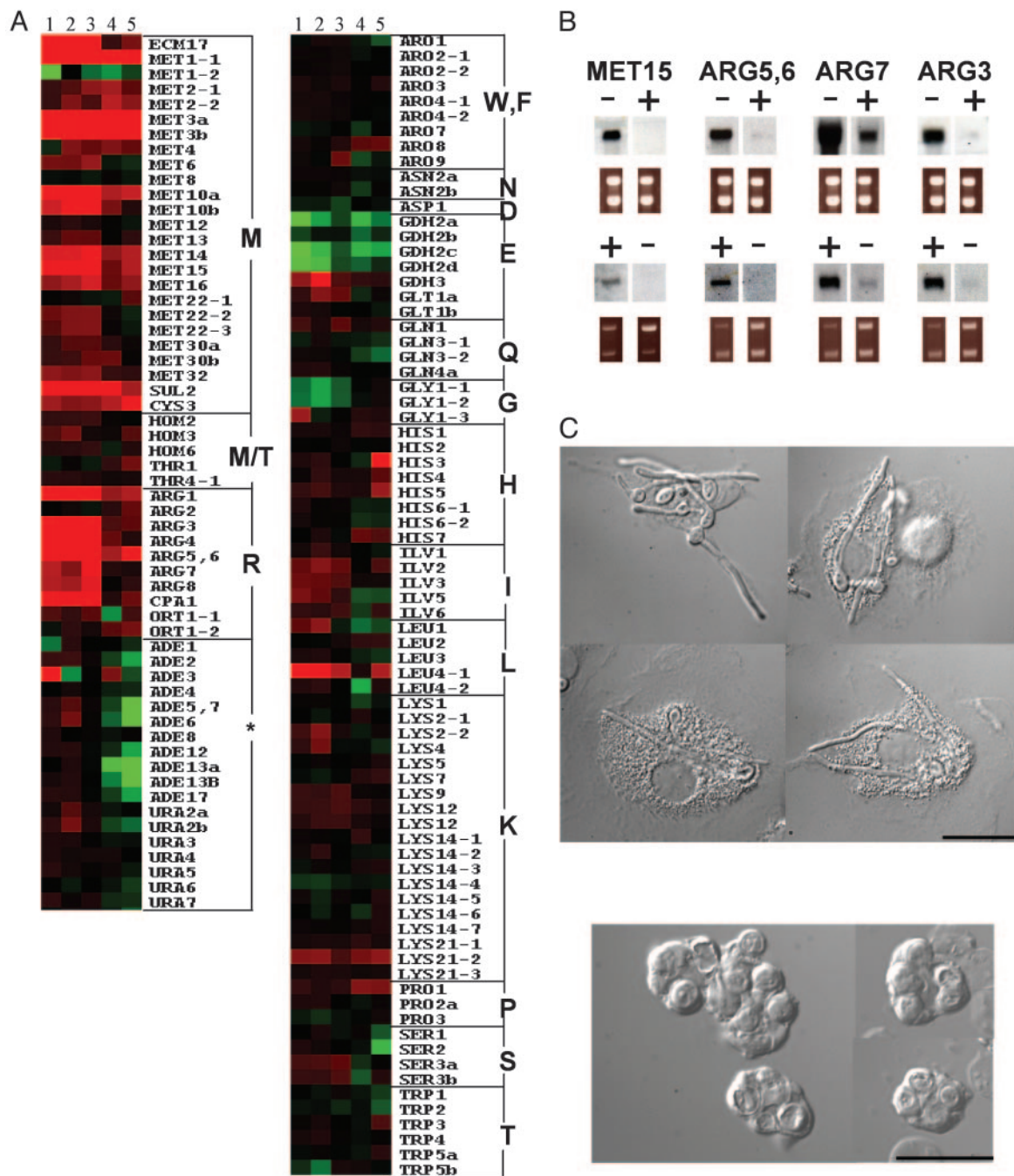


Fig. 4. Response of *Candida* to ingestion by neutrophils. In *Candida* the response to ingestion by neutrophils is similar to that engendered by amino acid deprivation. (A) *Candida* was cultured with neutrophils in RPMI medium 1640 containing 10% fresh human serum for 60 (column 5) or 80 (column 4) min or in minimal medium for 40 (column 3), 60 (column 1), or 80 (column 2) min. Amino acid and nucleic acid biosynthetic genes were selected for cluster analysis for illustration purposes. *ADE* and *URA* genes served as controls (*). Experiments were clustered by using CLUSTER and TREEVIEW. (B) *Candida* was cultured in minimal medium with (+) or without (-) amino acids (Upper) or in RPMI medium 1640 containing 10% fresh human serum with (+) or without (-) neutrophils (Lower) for 60 min. *Candida* mRNA was subjected to Northern analysis, using probes for the genes indicated. Ethidium bromide staining of ribosomal RNA to control for loading is shown in the lower gels. (C) *Candida* does not form filaments inside neutrophils. Yeast-form *Candida* cells were cultured with monocytes (Upper) or neutrophils (Lower) in RPMI medium 1640 containing 10% fresh human serum for 1 h. (Bar is 20 μ m.) See also Fig. 2, supplementary data at http://jura.wi.mit.edu/fink_public/neutrophils/.

methionine genes that were expressed in response to neutrophils are not in the *MET* cluster: *MET2*, *MET8*, *MET32*, *SUL1*, *SUL2*, nor are the arginine genes or the other biosynthetic genes *LEU1*, *SER3*, and *GLT1*. Third, of the 92 genes induced during S phase but not in the *MET* cluster there is only 1 gene (*SEO1*) that was also induced by neutrophils.

Neutrophils Also Induce the Expression of Amino Acid Genes in *C. albicans*. *Candida* was cocultured with neutrophils or grown in minimal medium, following the same procedures used for *Saccharomyces*. RNA preparations from the minimal medium and neutrophil experiments were used to hybridize to a cDNA microarray containing 11,000 sequences representing 7,600

ORFs (11). *Candida*, like *Saccharomyces*, responded to amino acid-deficient medium by up-regulating the methionine and arginine biosynthetic pathways (Fig. 4A, columns 1–3). These pathways were induced by *Candida* upon exposure to neutrophils in the presence of fresh human serum (Fig. 4A, columns 4 and 5). The gene for *GCN4*, which encodes the key transcriptional activator of amino acid biosynthetic genes, as well as that for *PCL5*, which regulates Gcn4p stability, were also induced in neutrophils. Northern analysis confirmed that four of the up-regulated amino acid genes identified by microarray analysis were also induced by amino acid deprivation (Fig. 4B Upper) and by neutrophils (Fig. 4B Lower).

Candida Phagocytosed by Neutrophils Responds to Oxidative Stress.

Candida exhibits a more obvious transcriptional antioxidant response to human neutrophils than does *Saccharomyces*. Six oxidation-related genes were highly induced by *Candida* in response to neutrophils: *SOD1* (8.8-fold), *CCP1-1* (11.55-fold), *CTA1-1* (43.8-fold), *CTA1-2* (7.4-fold), *GPX3-1* (11-fold), and *GPX3-2* (16.5-fold). *GPX3-3* was only mildly induced (2.6-fold), whereas *SOD2*, *CCP1-2*, and *GPX3-4* were not induced. *Candida* orthologs for *Saccharomyces CTT1* or *GPX2* were not detected in the *Candida* genomic database and therefore could not be examined for their induction in response to neutrophils.

Candida Does Not Form Filaments Inside Neutrophils. As reported previously, yeast-form *C. albicans* cells that are phagocytosed by macrophages switch to the hyphal form and escape the macrophages (12). Although we have observed this hyphal switch in human monocytes (Fig. 4C Upper), such switching did not occur inside human neutrophils (Fig. 4C Lower). After 1 h of exposure to human neutrophils, when 52% of the *C. albicans* cells were still alive, no *Candida* filaments were observed. At this time point, extensive filamentation was observed in the extracellular medium induced by serum (data not shown).

Monocytes Do Not Induce Amino Acid Biosynthesis. Phagocytosis of *Saccharomyces* by human monocytes (isolated from the same individual as neutrophils) did not induce the methionine and arginine biosynthetic pathways. The induction profile for *Saccharomyces* ingested by neutrophils was distinct from that of monocytes (Fig. 5, compare columns 1 and 2 with 3 and 4). Of the 83 yeast genes induced in response to neutrophils, 3 were consistently induced after exposure to monocytes: *YNR064C*, *STR3*, and *YER185W*. Because monocyte phagocytosis under these conditions was very efficient (90% of the monocytes ingested yeast after 40 min of incubation) the process of phagocytosis itself could not account for the induction of the amino acid genes observed in neutrophils.

Discussion

Our microarray experiments show that upon ingestion by neutrophils both *S. cerevisiae* and *C. albicans* induce the expression of genes from the methionine and arginine biosynthetic pathways. This transcriptional profile suggests that the microenvironment of the neutrophil compartment surrounding the microorganism is deficient in amino acids.

Previous analyses of the response of *Saccharomyces* to amino acids used two experimental conditions, starvation and deprivation. Because *Saccharomyces* has high internal pools of amino acids, amino acid starvation is achieved by reducing the internal pool of an amino acid by growth in the presence of an inhibitor of its biosynthetic pathway or by growth of a leaky auxotroph in the absence of the cognate amino acid (13, 14). Starvation for a single amino acid by either means increases the transcription of many diverse amino acid biosynthetic pathways (13, 14) and is mediated by the Gcn4 transcription factor. *C. albicans* also

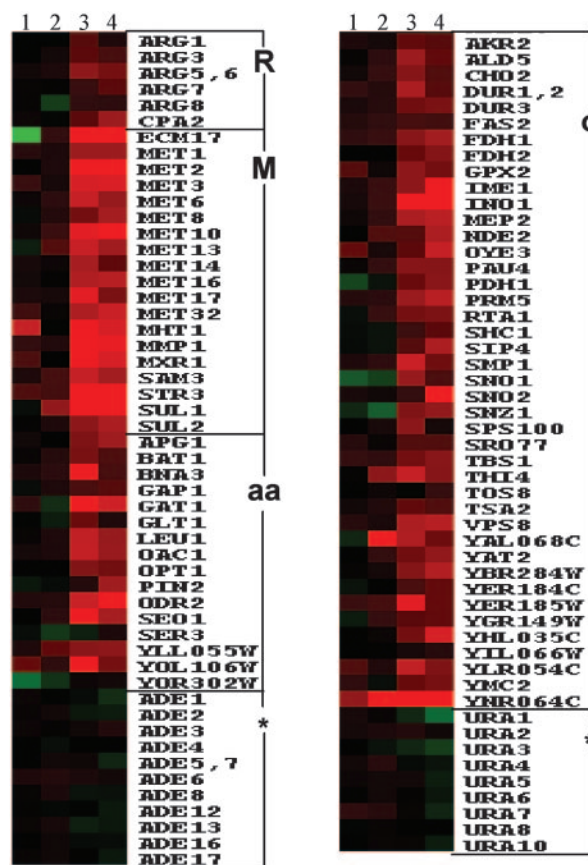


Fig. 5. Yeast methionine and arginine genes are induced in neutrophils, but not in monocytes. Yeast cells were cultured with neutrophils (columns 3 and 4) or monocytes (columns 1 and 2) in RPMI medium 1640 containing 10% fresh human serum for 40 min. All 83 genes meeting criteria for induction in neutrophils were selected for illustration purposes. Genes were divided into four groups: methionine (M), arginine (R), other amino acid genes (aa), and other genes (o). *ADE* and *URA* genes served as control (*) genes. Experiments were clustered by using CLUSTER and TREEVIEW.

appears to display this general control response to starvation (15).

Amino acid deprivation, caused by shifting *Saccharomyces* from a medium containing high levels of amino acids to one with no external amino acids, evokes a different response from that evoked by amino acid starvation. There is no general induction of many pathways. The only complete pathways induced by amino acid deprivation are those leading to the synthesis of methionine and arginine (6). The predominant transcriptional profile of both *Saccharomyces* and *Candida* inside neutrophils is most similar to that of amino acid deprivation, suggesting that the microenvironment of the neutrophil is poor in amino acids.

Our *in vitro* conditions, exposure to a medium containing amino acids (RPMI medium 1640) before encountering the immune cells, may represent a physiologically relevant environment because human blood and extracellular fluids, the sites of neutrophil–microbe interactions, contain appreciable concentrations of amino acids (16). When *Saccharomyces* was grown in medium poor in amino acids (HBSS), the methionine and arginine pathways were already maximally induced and failed to be further induced in the neutrophils. Group A streptococci also induce amino acid biosynthetic genes upon phagocytosis by human neutrophils, when grown in medium containing high concentrations of amino acids (17). When a similar experiment is performed with *Escherichia coli* and neutrophils in a medium

lacking amino acids (HBSS), no amino acid biosynthetic genes are induced (18).

Our results suggest that *Candida* has a much more robust response to oxidative stress than does *Saccharomyces*. Both Gram-positive [group A *Streptococcus* (17)] and Gram-negative [*E. coli* (18)] bacteria respond to neutrophil phagocytosis by induction of genes related to oxidative stress. This comparison suggests either that different organisms evoke distinct host cell responses or that different organisms respond differently to the same oxidative environment in the neutrophils.

The response of *Saccharomyces* and *Candida* to phagocytosis by neutrophils appears to be specific because these fungal cells do not show induction of the methionine or arginine pathways when efficiently phagocytosed by monocytes. In addition, the methionine and arginine biosynthetic genes are not induced when *Saccharomyces* is phagocytosed by the murine macrophage-like cell line J774A (1). *Salmonella enterica* ingested by macrophages also fails to induce amino acid genes (19). This difference between the immune cells suggests that the microenvironment of the monocyte phagosome may be sufficiently rich in amino acids to inhibit the induction of amino acid biosynthetic genes by phagocytosed yeast.

Differences between monocytes/macrophages and neutrophils in the biogenesis and composition of the phagosome could create distinct microenvironments unique to each immune cell. Upon ingestion the vacuolar pH of neutrophils becomes basic (20), whereas that of macrophages is acidic. Moreover, the macrophage phagosome appears to be derived predominantly from the endoplasmic reticulum (ER), whereas in neutrophils it is derived predominantly from plasma membrane components (21). This difference in phagosomal membrane origin could account for the difference in the microenvironment within the phagosomes of monocytes/macrophages and neutrophils. Neutrophils, whose phagosomal membranes are of plasma mem-

brane origin, might have amino acid transporters in a reversed orientation that would pump amino acids out of the phagosome and hence create an environment that is poor in amino acids. The phagosomes of monocytes or macrophages, whose phagosomal membranes are of ER origin, would not have such transporters and therefore would contain all of the amino acids internalized from the extracellular environment.

The difference in the transcriptional response of *Candida* to ingestion by monocytes and neutrophils could have biological consequences. Inside the macrophages *Candida* yeast-form cells form filaments (12), some of which pierce the macrophage and escape from the immune cell. By contrast, inside the neutrophil *Candida* remained in the yeast form and was ultimately killed. We did not observe induction of filaments, either germ tubes or hyphae, for up to 2 h after ingestion, a time at which a large proportion of the *Candida* cells are still viable. Whether this is a consequence of the amino acid free environment or other factors that differ between these two types of immune cells is not yet known.

We thank M. Lorenz for providing all PCR products for the *Candida* microarray, and for helpful discussions; J. Bender for technical advice; T. Volkert (Whitehead Institute Center for MicroArray Technology) for assistance in printing microarrays; G. Bell (Whitehead Institute Bio-computing) for help with presentation; G. Acton and E. Lander for access to microarray facilities; R. Wheeler and N. Hacohen for comments on this manuscript; and members of the Fink laboratory for their many valuable contributions. This work was conducted by using the W.M. Keck Foundation Biological Imaging Facility at the Whitehead Institute with the help of Nicki Watson. This work was supported by The Ellison Foundation (to G.R.F.); National Institutes of Health RO1 Grants GM 35010 (to I.R.-B.) and GM 40266 (to G.R.F.); the Morin Trust (to I.R.-B.); Clinical Investigator Training Program: Harvard/Massachusetts Institute of Technology Health Sciences and Technology—Beth Israel Deaconess Medical Center, in collaboration with Pfizer, Inc., and Grant IRG-87-007-13 from the American Cancer Society (to I.F.).

1. Lorenz, M. C. & Fink, G. R. (2001) *Nature* **412**, 83–86.
2. McKinney, J. D., Höner zu Bentrup, K., Muñoz-Eliás, E. J., Miczak, A., Chen, B., Chan, W. T., Swenson, D., Sacchetti, J. C., Jacobs, W. R., Jr., & Russell, D. G. (2000) *Nature* **406**, 735–738.
3. Rude, T. H., Toffaletti, D. L., Cox, G. M. & Perfect, J. R. (2002) *Infect. Immun.* **70**, 5684–5694.
4. Idnurm, A. & Howlett, B. J. (2002) *Eukaryot. Cell* **1**, 719–724.
5. Meshulam, T., Levitz, S. M., Christin, L. & Diamond, R. D. (1995) *J. Infect. Dis.* **172**, 1153–1156.
6. Gasch, A. P., Spellman, P. T., Kao, C. M., Carmel-Harel, O., Eisen, M. B., Storz, G., Botstein, D. & Brown, P. O. (2000) *Mol. Biol. Cell* **11**, 4241–4257.
7. Wodicka, L., Dong, H., Mittmann, M., Ho, M. H. & Lockhart, D. J. (1997) *Nat. Biotechnol.* **15**, 1359–1367.
8. Babior, B. M., Kipnes, R. S. & Curnutte, J. T. (1973) *J. Clin. Invest.* **52**, 741–744.
9. Causton, H. C., Ren, B., Koh, S. S., Harbison, C. T., Kanin, E., Jennings, E. G., Lee, T. I., True, H. L., Lander, E. S. & Young, R. A. (2001) *Mol. Biol. Cell* **12**, 323–337.
10. Spellman, P. T., Sherlock, G., Zhang, M. Q., Iyer, V. R., Anders, K., Eisen, M. B., Brown, P. O., Botstein, D. & Futcher, B. (1998) *Mol. Biol. Cell* **9**, 3273–3297.
11. Bennett, R. J., Uhl, M. A., Miller, M. G. & Johnson, A. D. (2003). *Mol. Cell Biol.*, in press.
12. Lo, H. J., Kohler, J. R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A. & Fink, G. R. (1997) *Cell* **90**, 939–949.
13. Natarajan, K., Meyer, M. R., Jackson, B. M., Slade, D., Roberts, C., Hinnebusch, A. G. & Marton, M. J. (2001) *Mol. Cell. Biol.* **21**, 4347–4368.
14. Hinnebusch, A. G. & Natarajan, K. (2002) *Eukaryot. Cell* **1**, 22–32.
15. Tripathi, G., Wiltshire, C., Macaskill, S., Tournu, H., Budge, S. & Brown, A. J. (2002) *EMBO J.* **21**, 5448–5456.
16. Cynober, L. A. (2002) *Nutrition* **18**, 761–766.
17. Voyich, J. M., Sturdevant, D. E., Braughton, K. R., Kobayashi, S. D., Lei, B., Virtaneva, K., Dorward, D. W., Musser, J. M. & DeLeo, F. R. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 1996–2001.
18. Staudinger, B. J., Oberdoerster, M. A., Lewis, P. J. & Rosen, H. (2002) *J. Clin. Invest.* **110**, 1151–1163.
19. Eriksson, S., Lucchini, S., Thompson, A., Rhen, M. & Hinton, J. C. (2003) *Mol. Microbiol.* **47**, 103–118.
20. Reeves, E. P., Lu, H., Jacobs, H. L., Messina, C. G., Bolsover, S., Gabella, G., Potma, E. O., Warley, A., Roes, J. & Segal, A. W. (2002) *Nature* **416**, 291–297.
21. Gagnon, E., Duclos, S., Rondeau, C., Chevet, E., Cameron, P. H., Steele-Mortimer, O., Paiement, J., Bergeron, J. J. & Desjardins, M. (2002) *Cell* **110**, 119–131.