Dissecting Colony Development of *Neurospora crassa* Using mRNA Profiling and Comparative Genomics Approaches

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Colony development, which includes hyphal extension, branching, anastomosis, and asexual sporulation, is a fundamental aspect of the life cycle of filamentous fungi; genetic mechanisms underlying these phenomena are poorly understood. We conducted transcriptional profiling during colony development of the model filamentous fungus *Neurospora crassa*, using 70-mer oligonucleotide microarrays. Relative mRNA expression levels were determined for six sections of defined age excised from a 27-h-old *N. crassa* colony. Functional category analysis showed that the expression of genes involved in cell membrane biosynthesis, polar growth, and cellular signaling was enriched at the periphery of the colony. The relative expression of genes involved in protein synthesis and energy production was enriched in the middle section of the colony, while sections of the colony undergoing asexual development (conidiogenesis) were enriched in expression of genes involved in protein/peptide degradation and unclassified proteins. A cross-examination of the *N. crassa* data set with a published data set of *Aspergillus niger* revealed shared patterns in the spatiotemporal regulation of gene orthologs during colony development. At present, less than 50% of genes in *N. crassa* have functional annotation, which imposes the chief limitation on data analysis. Using an evolutionary approach, we observed that the expression of phylogenetically conserved groups of genes was enriched in the middle section of an *N. crassa* colony whereas expression of genes unique to euascomycete species and of *N. crassa* orphan genes was enriched at the colony periphery and in the older, conditating sections of a fungal colony.

A major challenge of the postgenome era is the assignment of functional annotation data to genes within a genome to facilitate the ultimate goal of elucidating the mechanisms of developmental processes that orchestrate the particular life cycle of an organism. Studies of filamentous fungi have a major advantage in this endeavor over those of many metazoan species, since their genomes are small and haploid and propagation and archival storage are simple. Importantly, filamentous fungi also provide an opportunity to study multicellularity, which is lacking from the model eukaryotic unicellular yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*.

At present, more than 40 genome sequences for filamentous ascomycete species are available. Efforts aimed at gene prediction and functional annotation of genes have been undertaken, yet as many as half of the predicted protein coding genes (PCGs) in these species lack functional annotation. In addition, the vast majority of functional annotation associated with genes in filamentous ascomycete species has been assigned based solely on sequence or motif homology with known proteins, mainly based on experimental data for the model yeasts; biological function in these multicellular fungal organisms is uncertain.

*Neurospora crassa* has been studied for more than 80 years and is perhaps the best-understood filamentous fungus; its biochemistry, physiology, and genetics have been well characterized (18). *N. crassa* displays multicellularity and a complicated life cycle; it produces at least 28 morphologically distinct cell types (8, 9). There are ca. 10,000 predicted PCGs in the *N. crassa* genome (26). At present, 56% of these genes lack functional annotation.

One way to assess gene function is to generate mutant strains and evaluate phenotypes. Using a homologous recombination-based gene displacement method, Colot and coworkers created more than 7,000 deletion strains, corresponding to 4,922 unique genes in *N. crassa* (http://www.dartmouth.edu/~neurosporagenome/knockouts_completed.html). Phenotypic characterization for those gene deletion strains is being carried out by the scientific community (17, 19). One drawback of this methodology is that genes essential for viability cannot be evaluated. In addition, only a small proportion of mutants display perceivable phenotypic changes under standard laboratory conditions (33, 78). An alternative approach is to conduct screens for mutants that show phenotypic alterations. Such an approach identified defective genes in 45 temperature-sensitive mutants that resulted in phenotypic changes in cellular polarity and hyphal morphogenesis (61).

Another way to assess gene function is to use information obtained through transcriptome analysis, which evaluates the expression pattern of each gene upon alterations in experimental conditions or developmental stages. Recently we constructed 70-mer oligomer microarrays for *N. crassa*. The *N. crassa* microarrays have been demonstrated to be an important tool for the study of fungal development (35) and stress responses (70). In order to gain insight into gene function and to better understand genetic mechanisms underlying developmental programs in *N. crassa*, we investigated mRNA profiles.
associated with colony establishment. Fundamental colony development events in filamentous fungi, such as hyphal extension, septation, branching, anastomosis, and asexual sporulation, were recapitulated during *N. crassa* colony development. We cross-examined spatiotemporal patterns of gene expression during asexual colony development with functional category analysis of genes to elucidate biological processes participating during colony development. We observed an enrichment in mutant growth and polarity phenotypes in genes with functional annotation and which showed maximum expression at the colony periphery; many of these genes were previously identified in the above-described screen to isolate polarity and hyphal morphogenesis mutants (61). Thus, we predict that mRNA profiling data will provide hypotheses regarding gene function and will facilitate phenotypic screening of mutant phenotypes.

More than half of the predicted *N. crassa* genes lack functional annotation and fall into the category of encoding "unclassified proteins." We explored an alternative method for analysis, which does not rely on functional annotation (unpublished data). We determined the lineage specificity (LS) of each *N. crassa* gene, which describes the phylogenetic distribution of that gene’s homologs in related species (14). *N. crassa* genes were classified into six mutually exclusive LS groups using the SIMAP (similarity matrix of proteins) database (2, 55): (i) Euk/Prok-core (genes with homologs in non-fungal eukaryotes and/or prokaryotes), (ii) Dikarya-core (genes with homologs in basidiomycete and ascomycete species), (iii) Ascomycota core (genes with homologs in hemiascomycete species), (iv) Euroas-specific (genes with homologs found only in eusacymycete fungi), (v) *N. crassa* orphan genes, and (vi) others (gene homologs identified in prokaryotes, non-fungal eukaryotes, or basidiomycetes but not in ascomycete species except for *N. crassa*). We observed an enrichment for Euroas-specific genes with maximal expression at the colony periphery and during asexual development, while an enrichment for the Euk/Pro-core genes was associated with expression in the interior of the colony prior to conidiation. In this report, we analyze mRNA profiling data using functional category analysis and a phylogenetic approach to interpret the interplay of gene regulation and colony development in filamentous ascomycete fungi.

**MATERIALS AND METHODS**

**Construction of *N. crassa* oligonucleotide microarrays.** We developed oligonucleotide microarrays for the *Neurospora* research community using an NIH program project grant (GM068087). 70-mer oligonucleotide probes were designed using the ArrayOligoSelector software program (12), and the approximately 10,500 open reading frames derived mainly from the *N. crassa* databases at the Broad Institute (http://www.broad.mit.edu/annotation/fungi/Neurospora_crassa/;index.html) and MIPS (http://mips.gsf.de/genre/proj/ncrassa/). oligomers were immobilized on each of the gamma amino propyl surfaces. When using the 2500 oligomers in both the lineage and functional categories, the *N. crassa* microarray slides are available to the research community from the Fungal Genetics Stock Center (http://www.fgsc.net/). Information on the oligonucleotide gene set is available at the *Neurospora* Functional Genomics Database (http://www.yale.edu/townsend/Links/fdbase/introduction.html).

**Strain and culture conditions.** The wild-type laboratory strain, FGSC2489, used for the *N. crassa* gene project, was grown on Vogel’s minimal medium slant (77) at 25°C for 7 days. Growth under constant light conditions (ca. 300 lx) was performed to suppress synchronous gene expression associated with circadian rhythms (D. Bell-Pederson, personal communication). Conidia were resuspended in water, and the concentration was estimated using a hemocytometer. Approximately 1 × 10⁷ conidia were subsequently spread onto a Nunc 500-mm² square bioassay dish (Fisher Scientific) containing 150 ml of Bird’s medium (47) overlaid with a cellophane sheet (catalog no. 100357-652; VWR) (15). A detailed protocol can be found elsewhere (Large Plate Protocol; http://www.yale.edu/townsend/Links/fdbase/downloads.html). The culture was incubated at 25°C with constant light for 24 h. This yielded a homogenous mycelium lawn. A narrow cellophane strip (~0.5 cm by 22 cm) was excised and laid across the middle of fresh bird’s medium overlaid with cellophane in a Nunc square bioassay dish. The culture was incubated at 25°C with constant light, for 27 h. At every 3 h, the mycelial growth front was marked. After 27 h of incubation, five 1-cm-wide strips were excised from the plate. From the colony periphery inwards, strips corresponded to mycelia of 0 to ~3-h-old (referred to hereinafter as 3-h sample), 6 to ~9-h-old (referred to as 9 h), 12 to ~15-h-old (referred to as 15 h), 18 to ~21-h-old (referred to as 21 h), and 24 to ~27-h-old (referred to as 27 h). We defined the age of a section of a *N. crassa* colony as the time that elapsed from when hyphal tips were at the colony periphery; regardless of the duration of time since the point of inoculation, the colony periphery is always defined as 0 h old. The samples were kept at ~70°C until RNA extraction. A sample of 0.1 ml each of 1-h-old mycelia (1 h; 3-mm-width including the tip) was excised from a separate mycelial mat.

**RNA extraction and cDNA labeling.** Zirconia/silica beads (0.2 g, 0.5-mm-diameter; Biospec Products) and 1 ml of TRIZol reagent (Invitrogen Life Technologies) were added to 6 cm² of a frozen mycelia/cellophane strip. Cells were disrupted using a MiniBeadBeater instrument (Biospec Product) at maximum speed for 30 s twice. Total RNA was extracted according to the manufacturer’s protocol for TRIZol (Invitrogen). A 100-µg sample of total RNA was further cleaned using the RNeasy mini protocol for RNA cleanup (Qiagen). For cDNA synthesis and labeling, the ChipShot indirect labeling and clean-up system (Perkin-Elmer Corporation) was used. Briefly, 10 µg of total RNA was mixed with 1.1 ng of ArrayControl single RNA spike mixture (Ambion), and cDNA synthesis was carried out without random primers according to the manufacturer’s suggested protocol. The cDNA was purified using a ChipShot membrane column. The Cy3 and Cy5 cyanine dyes were incorporated into cDNA by adding Cy3 or Cy5 mono-V-hydroxysuccinimide ester dye (Amersham) to the cDNA solution for 1 h at 25°C. The cDNA was then cleaned by a ChipShot membrane column, vacuum dried, and subsequently used for hybridization.

**Hybridization and image acquisition.** The Pronto! hybridization kit (Corning Life Sciences) was used for prehybridization, hybridization, and washing according to the manufacturer’s suggested protocol (details can be found elsewhere [70]). An Axon GenePix 4000B scanner (Axon Instruments) was used to acquire images, and GenePix Pro 6 software was used to quantify hybridization signals. Bad spots were flagged automatically by the GenePix software, and subsequently each slide was inspected manually.

**Microarray experimental design and data analysis.** A closed-circuit design was used for microarray comparisons (Fig. 1). When a localized high background level was detected on a scanned microarray image, we eliminated probes within that region from further data analysis and repeated the hybridization. Thus, more than two hybridizations were made for each comparison. For comparisons made by three or more hybridizations each, biological replicates were included (Fig. 1). Because our growth conditions were highly controlled, observed variation between biological controls was negligible. Circuit designs for microarrays are statistically robust and improve resolution in identifying differentially regulated genes as compared to designs for microarrays that use a universal reference (36, 72, 82). In order to detect small differences in relative expression levels, most similar experimental conditions, e.g., neighboring time points, were preferentially compared to each other directly.

Hybridized spots in which at least one of the mean fluorescence intensities for Cy3 or Cy5 was greater than the mean background intensity plus three standard deviations of background intensity were scored for further analysis if fewer than 2% of pixels were saturated. Intensity data for Cy3 and Cy5 fluorescent dyes were then subjected to global normalization. Normalized ratio data were then analyzed using the BAGEL (Bayesian analysis of gene expression levels) software program, with which we inferred a relative gene expression level and credible interval for each gene at each experimental time point (73). Estimated gene expression profiles were grouped using a hybrid clustering method, hierarchical ordered partitioning and collapsing hybrid (HOPACH) (75), in which similarity in expression patterns between genes is measured as Pearson's correlation coefficient. Obtained expression clusters were visualized using the hierarchical clustering tool in the Exploer 3.5 software program (62). The microarray data have been deposited at the Yale Microarray Experimental Design site (http://www.yale.edu/townsend/Links/fdbase/introduction.html). Page 1 of Table S1 in the supplemental material lists mRNA profiling results, functional annotations, and LS groups of target genes.
RESULTS

A global transcriptional profile across a filamentous fungal colony. Neurospora crassa is an exceptional model with which to dissect fungal mechanisms of polar growth, colony development, and asexual reproduction. At 25°C on a solid medium, *N. crassa* hyphae show extreme polarity and extend ca. 0.3 cm/h or 7 cm/day; vegetative hyphae grow by polar expansion of hyphal compartments at the apex (29, 30). Three morphologically different types of hyphae make up the body of a fungal colony (8). Leader hyphae are located at the colony periphery and subsequently undergo subapical branching to form the majority of the fungal biomass. Trunk hyphae are found in the interior of a colony and are usually highly vacuolated with plugged septa. Fusion hyphae are involved in anastomosis events, which occur throughout the colony but are notably absent from hyphae at the colony tips (13, 28). During asexual development, aerial hyphae grow up from the surface of the colony and into the air. Aerial hyphae form the base of macroconidiophores, which are involved in the production of asexual spores, called macroconidia (referred to hereinafter as conidia) (18, 64). In *N. crassa*, macroconidia form by successive budding from the tips of aerial hyphae, which results in the formation of conidial chains. Thus, by assessing transcriptional profiles of genes across a fungal colony, we predicted that mRNA profiles for many tissue-specific and developmental stage-specific genes would be obtained, in addition to mRNA profiles of ubiquitously expressed genes.

To test this hypothesis, we isolated RNA from discrete sections of an *N. crassa* colony: 1-, 3-, 9-, 15-, 21-, and 27-h-old sections (Fig. 1A). Approximately 4 cm behind the hyphal tips (12 h old), the development of aerial hyphae was first observed. Conidial chains were observable in the 15-h-old section of the colony. High-density conidial chains and fully developed orange-pigmented conidia were profuse in hyphae 24 to 27 h old. Total RNA samples, obtained from each of the colony sections, were reverse transcribed, and subsequently competitive hybridizations were performed using full-genome *N. crassa* oligonucleotide microarrays. A closed-circuit design (Fig. 1B) was used for experimental procedures, and the relative expression level at each time point for each gene and credible intervals was inferred by the BAGEL software program (73). The closed-circuit design allows direct comparison of cDNA samples with smaller variance than with a more traditional reference sample design (36, 72, 82).

Of the 9,348 oligonucleotides on the microarrays representing predicted PCGs in *N. crassa* (http://mips.gsf.de/genre/proj/nccrassa/), 4,588 yielded mRNA profiles with statistical support for expression profiles (73). The remaining 4,760 oligonucleotides did not yield sufficient hybridization signals for the inference of relative expression levels across the colony sections. Some portion of these predicted genes are likely to be sexual phase specific and thus would not be expressed within a vegetative colony. Also, some genes could be transiently expressed during colony development; such genes would not pass the test for statistical support. We observed a correlation between detectability of transcripts and phylogenetic conservation of gene homologs. Out of the 2,421 *N. crassa* genes that are highly conserved among both eukaryotic and prokaryotic species, expression of 1,364 genes (56%) was detected (see page 1 of...
Berlin, Sachs, and Yanofsky identified a conidium-specific gene, con-10, that was highly expressed during conidiation (7, 58). We detected con-10 expression, with maximal expression at 27 h. Similarly, clock-controlled gene-2 (ccg-2; synonym, eas), which encodes a hydrophobin required for the formation of surface rodlets on macroconidia (5), showed maximal expression at 27 h. Thus, our mRNA profiling data faithfully recapitulated known gene expression data with morphological observations of the process of asexual reproduction. Note that all the colony sections were harvested at the same time of day. Because N. crassa was grown under constant light, neither of the clock-controlled genes ccg-1 and ccg-2 displayed circadian rhythm-controlled expression patterns; rather, they displayed developmental stage-specific patterns.

To identify clusters of coexpressed genes, we employed the HOPACH clustering methodology, which has been shown to partition genes into reliable and reproducible clusters (42, 75). Analysis of mRNA profiles with HOPACH revealed six distinctive clusters (Fig. 3B and C). The 1A cluster contains genes with maximal expression in the 1-h-old section of the colony (the front 3 mm of the colony periphery) but which otherwise showed lower relative expression levels in the remainder of the colony. Genes within the 1B cluster also showed maximal expression at 1 h; however, this group of genes also showed a second peak of expression at 21 h. Genes in the 3M, 9M, 21M, and 27M clusters showed maximal expression in the 3-, 9-, 21-, and 27-h-old sections of the fungal colony, respectively. As shown in Fig. 3, most genes showed either maximal or minimal relative expression levels in the 1-h-old sample. For example, genes in the clusters 1A and 1B had maximal expression levels at 1 h (Fig. 3B), while genes in the 9M and 21M clusters had minimal expression levels at this same time point. Genes in the 3M and 27M clusters showed moderate expression levels at the hyphal tip in comparison to relative expression levels in the rest of the colony. Note that the HOPACH algorithm does not take into account the credible interval at each time point for each gene, which is estimated by BAGEL. Hence, the expression clusters can contain genes for which the Bayesian statistics failed to detect differential expression. We therefore conducted an enrichment test to test the biological relevance of HOPACH clusters.

Analysis of clustered genes via FunCat classification revealed major cellular events associated with colony development. Genes participating in the same biological process are likely to be coregulated and are therefore more likely to belong to the same expression clusters (21). FunCat, developed by MIPS (44, 56), was used to test this hypothesis. FunCat is a hierarchically structured annotation scheme for the functional description of proteins. FunCat consists of 28 main functional categories (or branches) that cover general fields. Under the main functional categories, there are 1,362 subcategories, and they exhibit a hierarchical, tree-like structure with up to six levels of increasing specificity. In the MIPS FunCat scheme, a single PCG can have multiple functional categories at the same specificity level. Functional category analysis was followed by analysis for statistical enrichment for every functional category in each of the expression profile clusters.

Genes predicted to be within a particular functional category were not evenly distributed among the six expression clusters (Table 1). The 1,410 genes in the 1A cluster showed maximal expression levels of mRNA transcripts obtained by BAGEL. An asymmetric 95% credible interval for each expression level is also shown. The major protein of the Woronin body (69). (B) Actin gene act. (C) Clock-controlled gene (ccg-1), also known as glucose-repressible gene (ggr-1) (41, 46). (D) Conidiation gene (con-10) (7, 58). (E) Clock-controlled gene (ccg-2), also known as the fungal hydrophobin eas (5).

Table S1 in the supplemental material). However, out of the 1,640 genes found only in the N. crassa genome (N. crassa orphans), expression profiles were detected for only 449 (27%). It is possible that N. crassa orphan genes are expressed only under a limited set of environmental and developmental conditions which were not represented by growth under laboratory conditions. Alternatively, it is likely that some of the gene models within the N. crassa orphan group are bioinformatic artifacts. A great majority (98%) of N. crassa orphans lack functional annotation (unclassified genes). Underrepresentation of unclassified genes has been previously been observed in EST libraries (35).

Comparison of microarray data to previously identified differentially expressed marker genes. Tay and coworkers (69) examined levels of transcripts from the hexagonal-1 (hex-1), actin (act), and clock-controlled gene-1 (ccg-1) genes in an N. crassa colony and observed that expression of act and hex-1 was highly enriched at the leading edge of the fungal colony whereas ccg-1 (41, 46) showed maximal expression in a 10- to 12-h-old section of a colony (3.3 to 4.2 mm behind the periphery). Our microarray analysis faithfully reproduced these results (Fig. 2A, B, and C). HEX1 is a major structural component of Woronin bodies and is required for septal plugging upon catastrophic injury; the Woronin body is formed via the peroxisomal pathway (34, 68). Interestingly, 13 out of 17 genes predicted to encode peroxisomal proteins, including hex-1, showed maximal expression at the colony periphery (see page 2 of Table S1 in the supplemental material).
relative expression levels at the colony periphery compared to expression levels in the rest of the colony. A statistically significant enrichment for genes within the 1A cluster belonged to “Lipid, fatty acid, and isoprenoid metabolism (01.06)” under the main category “Metabolism (01)” (Table 1); 83 genes belonged to this category (see page 3 of Table S1 in the supplemental material). This gene set includes stt-4 (NCU09367; encoding a predicted 1-phosphatidylinositol 4-kinase), mss-4 (NCU02295; encoding a predicted phosphatidylinositol-4-phosphate 5-kinase), ire-1 (NCU02202; encoding a predicted serine/threonine kinase associated with endoplasmic reticulum quality control), gpi-3 (NCU09757; encoding a glycosylphosphatidylinositol anchor N-acetylgalcosamine transferase gene [11]), hmg-1 (NCU00712; encoding a predicted hydroxymethylglutaryl-coenzyme A [CoA] reductase), and erg-13 (NCU03922; encoding predicted hydroxymethylglutaryl-CoA synthase). Interestingly, strains containing mutations in any of these six genes display polarity defects, such as highly branched hyphae or apolar branches (10, 61). An enrichment among genes in cluster 1A was also observed for the main FunCat category “Cellular communication/Signal transduction mechanism (30)” and one of its subcategories, “cellular signaling (30.01)”; 50 genes in the 1A cluster belonged to this subcategory (see page 4 of Table S1 in the supplemental material). Genes encoding G proteins, protein kinases, and protein phosphatases predominate in this subcategory. For example, cot-1 (NCU07296; encoding a protein kinase) and cdc-42 (NCU06454; encoding a Rho-type GTPase) mutants both display polarity and growth directionality phenotypes (60, 65). Genes for the main category “Cell fate (40)” and its subcategory “Cell growth/morphogenesis (40.01)” were also enriched; 24 genes belong to this enriched category (see page 5 of Table S1 in the supplemental material). Examples are a homolog in N. crassa of the S. pombe tea1 gene (NCU00622); Tea1p localizes at the cell ends and is involved in polarized growth (4). Aspergillus nidulans strains that contain a mutation in the tea1 ortholog (tea4) show a zigzag

FIG. 3. Partitioning of microarray data into similar transcriptional profiles. (A) Alphanumerically ordered genes along the column (n = 4,588). From the left, column samples are as follows: 1 h, 3 h, 9 h, 15 h, 21 h, and 27 h old. Each gene’s expression values were standardized to have a mean of 0 and standard deviation of 1 across the six samples. A lighter color in the figure is correlated with a higher expression level. (B) Genes partitioned into six expression clusters by using the HOPACH algorithm. (C) Average expression contour of genes within each of the clusters. To obtain each profile, the sum of each gene’s expression values across the six samples was standardized to 1. Next, values for each of the six samples for all the genes in each cluster were summed, and the summed value for the six samples for each cluster was scaled to 1. A schematic representation of colony development is shown at the bottom. Hyphae were inoculated at the right corner, and fungus is growing leftward. Development of aerial hyphae is observed in the ~15-h-old section of the colony. At 27 h, long macroconidial chains developed. (D) Average expression counter for each HOPACH cluster of mRNA profiles of A. niger colony development (39). The colony diameter for A. niger was 5.5 cm after 7 days (bottom figure). mRNA samples were prepared from concentric zones of the circular colony. Ages of zones are 0 to 1, 1 to 2, 2 to 3, 3 to 4, and 4 to 7 days for zones Z5, Z4, Z3, Z2, and Z1, respectively.
hyphal growth pattern (67). Another example is a homolog of S. cerevisiae Spa2 (NCU03115); Spa2 is a component of polarisome and is required for establishment of cell polarity and pseudohyphal growth (27, 63). Recently Spa2 homologs in A. nidulans (76) and N. crassa (C. L. Araujo-Palomares, M. Riquelme and E. Castro-Longoria, presented at Neurospora 2008, Asilomar, CA, 27 to 30 March 2008) have been shown to localize to the Spitzenkörper at hyphal apices. Expression of genes within the functional category “Biogenesis of cellular components (42)” and its subcategory “actin cytoskeleton/structural proteins” were also enriched in cluster 1A (see page 6 of Table S1 in the supplemental material). Thus, our transcriptional profiling is consistent with cell biology associated with hyphal tip growth, polarity, and environmental sensing, which are major aspects of the function of hyphae at the colony periphery.

The 492 genes in the 1B cluster showed maximal expression

| Table 1. Enriched functional categories for each of the six HOPACH clusters |
|-----------------------------|-----------------------------|
| HOPACH cluster or enriched FunCat category | No. of genes\(a\) | \(P\) value\(b\) |
|-----------------------------|-----------------------------|
| **1A (1 h maximum; 1,410 genes)** | | |
| 01.06 Lipid, fatty acid, and isoprenoid metabolism | 83 | 57.5 | ** |
| 10.03.03 Cytokines (cell division)/septum formation and hydrolysis | 15 | 6.8 | * |
| 30 Cellular communication/signal transduction mechanism | 52 | 34.1 | ** |
| 30.01 Cellular signaling | 50 | 32.3 | * |
| 40 Cell fate | 28 | 15.7 | ** |
| 40.01 Cell growth/morphogenesis | 24 | 12.9 | * |
| 42 Biogenesis of cellular components | 57 | 37.2 | ** |
| 42.04 Cytoskeleton/structural proteins | 19 | 9.2 | * |
| 42.04.03 Actin cytoskeleton | 12 | 5.2 | * |
| 70.04 Cytoskeleton | 22 | 10.1 | ** |
| **1B (1 h and 21 h max, 492 genes)** | | |
| 99 Unclassified proteins | 291 | 233.9 | *** |
| **3M (3 h max, 456 genes)** | | |
| 70.02 Eukaryotic plasma membrane/membrane attached | 18 | 7.3 | * |
| **9M (9 to 15 h max, 1,178 genes)** | | |
| 01 Metabolism | 240 | 200.3 | ** |
| 01.01 Amino acid metabolism | 60 | 31.1 | *** |
| 01.01.03.05 Metabolism of arginine | 6 | 1.5 | * |
| 01.01.06 Metabolism of the aspartate family | 10 | 3.9 | * |
| 01.03.01 Purine nucleotide/nucleoside/nucleobase metabolism | 15 | 5.9 | ** |
| 02 Energy | 92 | 40.3 | *** |
| 02.01 Glycolysis and gluconeogenesis | 12 | 4.6 | * |
| 02.10 Tricarboxylic-acid pathway (citrate cycle, Krebs cycle, TCA cycle) | 15 | 5.4 | ** |
| 02.11 Electron transport and membrane-associated energy conservation | 33 | 12.6 | *** |
| 02.13 Respiration | 39 | 15.7 | *** |
| 02.13.03 Aerobic respiration | 14 | 6.2 | * |
| 02.45 Energy conversion and regeneration | 9 | 3.1 | * |
| 02.45.15 Energy generation (e.g., ATP synthase) | 9 | 2.6 | * |
| 12 Protein synthesis | 153 | 51.1 | *** |
| 12.01 Ribosome biogenesis | 107 | 32.4 | *** |
| 12.01.01 Ribosomal proteins | 76 | 21.3 | *** |
| 12.04 Translation | 23 | 9.2 | *** |
| 12.04.01 Translation initiation | 14 | 6.2 | * |
| 12.10 Aminoacyl-tRNA synthetases | 19 | 6.7 | *** |
| 20.01.15 Electron transport | 21 | 8.7 | *** |
| 34.01.01.03 Homeostasis of protons | 14 | 4.9 | ** |
| 70 Subcellular localization | 313 | 183.3 | *** |
| 70.03 Cytoplasm | 103 | 39.3 | *** |
| 70.16 Mitochondrion | 133 | 54.2 | *** |
| **21M (21 h max, 677 genes)** | | |
| 14 Protein fate (folding, modification, destination) | 73 | 50.6 | ** |
| 14.13 Protein/peptide degradation | 26 | 12.8 | * |
| 14.13.01 Cytoplasmic and nuclear protein degradation | 13 | 4.0 | ** |
| 99 Unclassified proteins | 361 | 321.8 | ** |
| **27M (27 h max, 375 genes)** | | |
| 99 Unclassified proteins | 204 | 178.3 | * |

\(a\) Observed number of genes and expected number of genes if probabilities of each outcome are independent of the cluster.

\(b\) \(P\) values were determined using Fisher’s exact test with Benjamini and Hochberg multiple testing correction. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\). Only enriched categories are shown.
at the colony periphery (0 to 1 h) and in the section of the colony initiating asexual development (21 h) (Fig. 3). The distribution of FunCat categories in the 1B cluster was very distinct from that in the 1A cluster; genes within the category “Unclassified proteins (99)” constituted the only functional category that showed statistical enrichment (Table 1). It is possible that genes in the 1B cluster may be involved in hyphal extension, which occurs in the periphery of the colony and also during the development of aerial hyphae. Further phenotypic analysis of strains containing mutations in genes within the 1B cluster is required to test this hypothesis.

The 456 genes within the 3M cluster showed maximal expression at 1 cm from the colony periphery (Fig. 3). An enrichment of only one entry “Eukaryotic plasma membrane/membrane attached (70.02)” of the subcategory “Subcellular localization (70)” was detected (Table 1). Membrane transporters of diverse functions dominate this group of genes (see page 1 of Table S1 in the supplemental material).

Genes encoding proteins associated with protein biosynthesis and energy production show maximal expression in the colony interior. The 1,178 genes within the 9M cluster showed maximal relative expression levels in the middle section of the N. crassa colony (9- and 15-h-old sections, spanning a 6- to 15-h-old part) and minimal relative expression levels in the periphery of the colony (Fig. 3). The functional categories that were enriched in the 9M cluster were very distinct from functional categories that were enriched in the 1A and 1B gene clusters (Table 1). In the 9M cluster, the main FunCat category “Metabolism (01)” and its subcategories “Amino acid metabolism (01.10)” and “Nucleotide/nucleoside/nucleobase metabolism (01.03.01)” were overrepresented. Also, genes within the functional category for “Energy (02)” and its subcategories “Glycolysis and gluconeogenesis (02.01),” “Tricarboxylic-acid pathway (02.10),” “Electron transport and membrane-associated energy conservation (02.11),” and “Respiration (02.13)” were enriched. Genes for “Protein synthesis (12)” and its subcategories “Ribosome biogenesis (12.01),” “Translation (12.04),” and “Aminoacyl-tRNA synthetases (12.10)” were also significantly overrepresented. Thus, the 6- to 15-h-old part of an N. crassa colony, 2 to 5 cm behind the colony periphery, was engaged in mass production of mRNAs for proteins predicted to be involved in protein synthesis, amino acid metabolism, and energy generation.

Enrichment of functional categories in sections of an N. crassa colony undergoing asexual development. The 21M cluster contained 677 genes that showed maximal expression at 21 h (Fig. 3). The process of asexual development (conidiogenesis) was profuse in this section of an N. crassa colony. Genes for “Protein fate (14)” and its subcategory “Protein/peptide degradation (14.13)” were enriched in the 21M cluster (Table 1). Of the 26 genes in the 21M cluster in the “Protein/peptide degradation” functional category, 11 genes encoded predicted proteasome-related proteins (see page 7 of Table S1 in the supplemental material). The main function of the proteasome is to degrade unneeded or damaged proteins. In Drosophila, genes encoding proteasomal proteins are under developmental control and are involved in cell proliferation and morphogenesis (37). It is possible that proteasomes in N. crassa are involved in reconstruction of hyphal cellular components required for the generation of conidiophores and macroconidia observed in this section of the colony. Genes within the 21M cluster also showed enrichment in the category “Unclassified proteins (99),” as was also observed in the 1B cluster, which contained genes that showed a second peak of expression at 21 h. It is likely that some of these unclassified genes are involved in aerial hypha extension and/or conidiogenesis.

The 375 genes within the 27M cluster showed maximal expression at 27 h (Fig. 3), as with the 1B cluster, “Unclassified proteins (99),” the only category enriched within the 27M cluster (Table 1).

Mutations in genes which show high expression levels at the colony periphery often display severe morphological phenotypes. Seiler and Plamann screened N. crassa for temperature-sensitive mutants defective in cellular polarity and hyphal morphogenesis (61). Mutations in 45 genes responsible for 21 distinct morphological classes of polarity defects were identified. Out of the 45 genes identified in that study, mRNA profiles for 28 were obtained from our vegetative growth transcriptional profiling. The expression pattern of the majority of these genes (20 out of 28) belonged to either the 1A or 1B clusters, both of which showed maximal expression at the colony periphery (Table 2). These data imply that mutations in genes having maximal expression at the colony periphery are more likely to result in strains with growth and polarity phenotypes. To test the hypothesis that mutations in genes that show maximal expression at the colony periphery are more likely to result in strains showing growth or polarity defects, the phenotypes which were available for 571 of the gene knockout mutant strains (www.broad.mit.edu/annotation/genome/neurospora/blastpage/Genes.html) were evaluated. Of the 571 genes mutated in these strains, we obtained mRNA profiles for 280 during vegetative growth (see page 1 of Table S1 in the supplemental material). Out of these 280 genes, 141 belonged to the 1A or 1B cluster, which included genes with maximal expression at the colony periphery. The remaining 139 genes belonged to the cluster 3M, 9M, 21M, or 27 M, which showed maximal expression in the inner part of the colony. Of the 141 genes that showed maximal expression at the colony periphery, mutations in 48 of them (34%) gave strains that showed slow-growth phenotypes (a linear growth rate of less than 60 mm/day) (Table 1). Interestingly, 14 of these predicted genes encode hypothetical, conserved hypothetical, or putative proteins. In contrast, of the 139 genes that showed maximal expression in the interior of the colony, mutations in only 30 (22%) gave strains that exhibited a slow-growth phenotype. Thus, mutations in genes that showed maximal expression at the colony periphery were more likely to result in growth impairment than mutations in genes whose maximal expression occurred in the interior of the colony (P = 0.02). These observations of mutant phenotypes support the peripheral zone hypothesis in which the hyphal distribution is governed by mechanisms operating locally at the hyphal apex (51, 74).

Conservation of expression timing in gene orthologs in N. crassa and Aspergillus niger during colony development. Recently a genome-wide expression analysis was conducted to assess spatial expression profiles in an A. niger colony (39). N. crassa, which is a member of the Sordariomycetes, and A. niger, a member of the Eurotiomycetes, are distantly related classes within the phylum Ascomycota. A comparison of transcriptomes between A. niger and N. crassa may therefore shed light
### TABLE 2. Phenotypes of strains containing mutations in genes which showed maximum expression at colony periphery

| Probe ID | Gene ID | MIPS ID | HOPACH profile | Gene name | A. niger ortholog | MIPS description | LS group | Phenotype | Linear growth rate |
|----------|---------|---------|----------------|-----------|-------------------|------------------|----------|-----------|-------------------|
| NCU00712.2 | 1nc70_130 | IA | 1D | bmg-1 | An07g08280 | Probable 3-hydroxy-3-methylglutaryl-CoA reductase | Dikarya-core | Highly branched | 0% |
| NCU01992.2 | b13d15_060 | IA | 1D | sec-21 | An07g06030 | Related to coamter gamma-2 subunit | Euk/Prok-core | Highly branched | 0% |
| NCU02202.2 | 7nc534_010 | IA | 3P | ire-1 | An01g06550 | IRE1 precursor | Dikarya-core | Thick, septated, and swollen | 0% |
| NCU02295.2 | 5123_140 | IA | 3P | ms-4 | An11g10410 | Related to phosphatidylinositol-4-phosphate 5-kinase | Dikarya-core | Highly branched | 0% |
| NCU02620.2 | 53h1_020 | IA | pod-4 | | | Conserved hypothetical protein | Euk/Prok-core | Bunches of grapes | 0% |
| NCU02829.2 | 1nc725_060 | IA | 1D | sec-53 | An18g06500 | Probable phosphomannomutase | Dikarya-core | Fast lysis | 0% |
| NCU03483.2 | b8112_160 | IA | ro-3 | | | RO-3 | Euk/Prok-core | Curled hyphae | 0% |
| NCU03628.2 | b11n2_160 | IA | pod-5 | | | Conserved hypothetical protein | Euk/Prok-core | Base of branch bulbous | 0% |
| NCU03912.2 | b23d11_320 | IA | 2D | erg-13 | An02g06320 | Probable hydroxymethylglutaryl-CoA synthase | Dikarya-core | Highly branched | 0% |
| NCU04121.2 | 9g6_370 | IA | 5P | apl-4 | An01g02600 | Probable gamma-adaptin | Euk/Prok-core | Fast lysis | 0% |
| NCU04173.2 | b7a16_050 | IA | 1D | act | An15g05650 | Actin | Euk/Prok-core | Tip branches | 0% |
| NCU04189.2 | b13c20_130 | IA | 2D | gsb-1 | An17g02120 | 1,3-Beta-glucan synthase | Euk/Prok-core | Highly branched | 0% |
| NCU04545.2 | 3nc220_410 | IA | 3P | cdc-42 | An02g14200 | Probable GTP binding protein | Euk/Prok-core | Zigzag growth | 0% |
| NCU07261.2 | 4nc600_110 | IB | alg-1 | | | Related to beta-1,4-mannosyltransferase | Euk/Prok-core | Highly branched | 0% |
| NCU07280.2 | 4nc605_020 | IB | 5P | ypk-4 | An02g05860 | Probable protein kinase 1 | Dikarya-core | Tip branches | 0% |
| NCU07296.2 | 4nc605_170 | IB | 5P | cot-1 | An01g07180 | Probable protein kinase 1 | Euk/Prok-core | Highly branched | 0% |
| NCU07312.2 | 4nc610_150 | IA | sec-27 | | | Related to phosophomannosyl-4-kine | Euk/Prok-core | Apolar branches | 0% |
| NCU07967.2 | b24g5_070 | IA | 5P | gpi-3 | An01g09910 | Related to N-acetylglucosaminyltransferase | Euk/Prok-core | Apolar branches | 0% |
| NCU08633.2 | b11o8_140 | IB | 1D | cop-1 | An08g06330 | Related to coamter epsilon subunit | Euk/Prok-core | Highly branched | 0% |

### KO mutants

| Probe ID | Gene ID | MIPS ID | HOPACH profile | Gene name | A. niger ortholog | MIPS description | LS group | Phenotype | Linear growth rate |
|----------|---------|---------|----------------|-----------|-------------------|------------------|----------|-----------|-------------------|
| NCU00406.2 | 3nc450_410 | IA | 3P | An17g02010 | Probable protein kinase CHM1 | Dikarya-core | 0% |
| NCU00927.2 | 20h10_040 | IA | 1D | putative fatty acid desaturase (mid) | Euk/Prok-core | 0% |
| NCU03593.2 | g229_010 | IA | kal-1 | | | Probable homeoprotein | Eua-specific | 0% |
| NCU07471.2 | 1nc130_160 | IA | 1D | | | Related to putative fatty acid desaturase (mid) | Euk/Prok-core | 0% |
| NCU08600.2 | b2221_090 | IA | 1D | xnc170_080 | An11g06620 | Probable rh3 protein | Dikarya-core | 0% |
| NCU08616.2 | b11n2_310 | IA | 2D | xnc170_080 | An05g00370 | Probable RAS-2 protein | Euk/Prok-core | 0% |
| NCU08631.2 | b2312_200 | IB | alg-1 | | | Related to coamter epsilon subunit | Dikarya-core | 0% |
| NCU08673.2 | 64c2_240 | IA | 1D | gmr-1 | An03g04120 | Probable chlorath-associated | Euk/Prok-core | 0% |
| NCU08685.2 | b315_150 | IA | 1D | Hypothetical protein | Eua-specific | 0% |
| NCU08722.2 | 3nc440_750 | IA | 7D | | | Related to cellulase homolog 4A | Euk/Prok-core | 0% |
| NCU08737.2 | 3nc190_300 | IB | 5P | | | Related to alpha-adaptin C | Euk/Prok-core | 0% |
| NCU08763.2 | 64c2_240 | IA | 1D | gmr-1 | An03g04120 | Probable chlorath-associated | Euk/Prok-core | 0% |
| NCU08785.2 | 7nc610_440 | IA | 3P | SPA2 | An02g07140 | Putative protein | Asco-core | 0% |
| NCU08791.2 | 1nc470_180 | IA | 3P | | | Related to SPA2 protein | Dikarya-core | 0% |
| NCU09993.2 | xnc170_080 | IA | 1D | | | Related to mitosis and maintenance of ploidy protein Mobi1 | Asco-core | 0% |
| NCU10498.2 | b7n4_100 | IA | | | | Related to Ser/Thr protein kinase IME | Euk/Prok-core | 0% |

Continued on following page
on the fundamental mechanisms governing colony development in filamentous fungi.

There is a large difference in the growth rate between *N. crassa* and *A. niger*; the linear growth rate of *A. niger* was 0.5 cm per day, in contrast to 7 cm per day for that of *N. crassa*. The diameter of the *A. niger* colony was 5.5 cm after 7 days. For the transcriptional profiling study (39), five concentric zones were excised from this colony, which represents approximately 1 day of growth for each section (Fig. 3D, zones 2 to 5) (39), with the exception of the most internal section of the colony (Z1), which represented ~3 days of growth. We used HOPACH clustering (75) to group the published *A. niger* mRNA profiles; six distinctive clusters were identified (Fig. 3D). The 1D cluster contains genes with maximal relative expression levels in the 1-day-old section of the colony, including the colony periphery. Genes within the 2D cluster showed maximal expression in the 2-day-old section of the *A. niger* colony, while genes in the 3P cluster showed an expression plateau in the 4- and 7-day-old sections of the colony. Similarly, genes in the 5P cluster showed an expression plateau in the 2- and 3-day-old sections of the colony. Genes within the 4P and 7D showed maximal expression in the 4- and 7-day-old sections of the *A. niger* colony, respectively.

A reciprocal BLAST search identified 5,175 gene orthologs.
between *N. crassa* and *A. niger*. mRNA profiles for 2,156 of the 5,175 gene orthologs were obtained for both *N. crassa* and *A. niger* during vegetative growth and colony establishment (see page 1 of Table S1 in the supplemental material). An enrichment analysis showed a correlation of mRNA profiles of *A. niger* and *N. crassa* during colony development (Table 3). For example, orthologs of genes in the *N. crassa* cluster 9M were found in the *A. niger* clusters 1D and 2D by 22% and 45% more than expected, respectively. An enrichment analysis of functional categories revealed that the intersection of the *A. niger* 1D and *N. crassa* 9M clusters was overrepresented for genes in “Amino acid metabolism (01.01),” the main category “Energy (02),” and its subcategories “Electron transport (02.11)” and “Respiration (02.13).” The intersection of the *A. niger* 2D and *N. crassa* 9M clusters was overrepresented with genes belonging to the main FunCat category “Protein synthesis (12)” and its subcategory “Ribosomal biogenesis (12.01).” The *A. niger* 1D cluster included genes that showed maximal expression in the outer ~1 cm of the colony. The *A. niger* 1D cluster was not enriched with orthologs from the *N. crassa* 1A cluster, which contained genes that showed maximal expression at the colony periphery (0- to 1-h-old hyphae). However, a closer scrutiny of the functional category data revealed an enrichment of common subcategories between the two organisms. For example, genes encoding proteins predicted to be involved in “Lipid, fatty acid and isoprenoid metabolism (01.06)” were overrepresented in the *A. niger* 1D and *N. crassa* 1A clusters. Of the 16 gene orthologs belonging to the intersection of the *N. crassa* 1A cluster and the *A. niger* 1D expression clusters, all showed maximal expression at the colony periphery (clusters 1A and 1D in Fig. 3 and Table 4) and six genes were involved in ergosterol biosynthesis. The remaining 10 orthologs are predicted to be involved in metabolism of lipids and fatty acids. Thus, the 16 genes that showed maximal expression at the colony periphery in both *N. crassa* and *A. nidulans* are predicted to be involved in membrane biogenesis. These data indicate that these orthologs are likely to be involved in the same biological processes at the colony periphery in the two fungi; they are functional orthologs.

In addition to an enrichment for the functional category “Lipid, fatty acid, and isoprenoid metabolism (01.06),” the functional category “Biogenesis of cellular components (42)” and its subcategory “Actin cytoskeleton (42.04.03)” were enriched. The intersection was enriched (P < 0.001). Within the intersection, the FunCat categories “01.01 Amino acid metabolism,” “02 Energy,” “02.11 Electron transport,” and “02.13 Respiration” were enriched.

**Evolutionary approach using lineage specificity groups casts new light on gene regulation during colony development.** At present, 56% of the predicted *N. crassa* PCGs lack functional annotation, which imposes the chief limitation on data analysis. We therefore explored an approach that does not rely on functional annotation to develop hypotheses about possible functions of unclassified genes whose expression was associated with colony development in *N. crassa*. First, *N. crassa*
TABLE 4. Gene orthologs expressed at colony periphery in both N. crassa and A. niger

| FunCat category or probe IDa | Gene ID       | HOPACH profile | MIPS ID | A. niger ortholog | MIPS descriptionb | LS group       |
|------------------------------|---------------|----------------|---------|-------------------|-------------------|----------------|
| 01.06 Lipid, fatty acid, and isoprenoid metabolism | NCU00302.2 | 1A 1D | 3nc442_160  | An08g03450 | Related to 3-ketosphinganine reductase | Euk/Prok-core |
| 3nc442_160_763           | NCU00712.2 | 1A 1D | 1nc570_130 | An07g08280 | Probable 3-hydroxy-3-methylglutaryl-CoA reductase | Dikarya-core |
| 1nc570_130_3150          | NCU02571.2 | 1A 1D | b5k2_130   | An16g09190 | Probable acetoacetyl-CoA thiolase (ERG10) | Asco-core |
| b5k2_130_987             | NCU02624.2 | 1A 1D | 1nc420_010 | An13g00090 | Probable cytochrome P450 51 (iburicol 14 alpha-demethylase) (ERG11) | Euasco-specific |
| 1nc420_010_629           | NCU03006.2 | 1A 1D | b24p7_240  | An14g01590 | Probable delta(24)-sterol c-methyltransferase (ERG6) | Asco-core |
| b24p7_240_262            | NCU03639.2 | 1A 1D | b11n2_060  | An09g02180 | Probable triacylglycerol lipase precursor | Euasco-specific |
| 3nc305_100_387           | NCU06207.2 | 1A 1D | 3nc305_100 | An15g00150 | Probable sterol delta 5,6-desaturase (ERG3) | Asco-core |
| 3nc305_100_387           | NCU06402.2 | 1A 1D | 4nc630_230 | An03g06410 | Probable C-4 methyl sterol oxidase (ERG25) | Dikarya-core |
| 4nc630_230_431           | NCU06694.2 | 1A 1D | 7f4_130    | An02g09910 | Probable fatty acid elongase (FEN1) | Asco-core |
| 7f4_130_376              | NCU06877.2 | 1A 1D | xnc064_040 | An06g01900 | Related to phosphatidylinositol transfer protein | Dikarya-core |
| xnc064_040_1825          | NCU07859.2 | 1A 1D | 3nc190_170 | An13g00740 | Related to cyclopropane-fatty-acyl-phospholipid synthase | Euk/Prok-core |
| 3nc190_170_444           | NCU07958.2 | 1A 1D | 3nc190_170 | An15g02860 | Probable 2,4-dienoyl-CoA reductase SPS19 | Asco-core |
| 3nc285_130_781           | NCU08280.2 | 1A 1D | 3nc348_010 | An03g03770 | Related to squalene monoxygenase (ERG1) | Dikarya-core |
| 3nc348_010_1332          | NCU08924.2 | 1A 1D | 3nc440_050 | An11g00400 | Related to acyl-CoA dehydrogenase, long-chain specific precursor | Dikarya-core |
| 3nc440_050_5513          | NCU09367.2 | 1A 1D | 3nc440_050 | An13g00110 | Related to phosphatidylinositol-4-kinase | Dikarya-core |
| 42 Biogenesis of cellular components | NCU00202.2 | 1A 1D | 3nc440_050 | An02g01210 | Probable coronin | Asco-core |
| 3nc440_050_1150          | NCU00243.2 | 1A 1D | 3nc440_460 | An02g07690 | Probable EB1-like protein | Euk/Prok-core |
| 3nc440_460_555           | NCU00495.2 | 1A 1D | 3nc460_150 | An01g14250 | Related to coatomer delta subunit (delta-coat protein) | Euk/Prok-core |
| 3nc460_150_1150          | NCU02781.2 | 1A 1D | 1nc700_170 | An18g06590 | Probable Arp2/3 protein complex subunit sop2 | Euk/Prok-core |
| 3nc700_170_1027          | NCU03407.2 | 1A 1D | b11b23_030 | An14g05530 | Rho-type GTPase, rhof4 | Euk/Prok-core |
| 29fb_090_2338            | NCU04404.2 | 1A 1D | 3nc120_030 | An08g03270 | Probable coatomer complex beta chain | Dikarya-core |
| 3nc120_030_906           | NCU05686.2 | 1A 1D | 3nc120_030 | An07g07530 | Probable cell wall protein UTR2 | Dikarya-core |
| 3nc295_010_4012          | NCU06171.2 | 1A 1D | 3nc295_010 | An13g00290 | Related to PAN1, actin cytoskeleton assembly protein | Dikarya-core |
| 3nc220_170_2025          | NCU06429.2 | 1A 1D | 3nc220_170 | An03g04570 | Related to alpha-actinin | Euk/Prok-core |
| 3nc220_340_125           | NCU06447.2 | 1A 1D | 3nc220_340 | An02g02830 | Probable RER1 protein | Euk/Prok-core |
| b14d6_340_237            | NCU06781.2 | 1A 1D | b14d6_340  | An08g07350 | Probable beta (1-3) glucanosyltransferase | Asco-core |
| 1nc130_160_634           | NCU07471.2 | 1A 1D | 1nc130_160 | An01g05290 | Probable F-actin capping protein beta subunit | Euk/Prok-core |

Continued on following page
genes were grouped into six mutually exclusive lineage specificity (LS) groups according to the phylogenetic distribution of their gene homologs (14). These six groups were the following: (i) Eukaryote/Prokaryote core (Euk/Pro-core), (ii) Dikaryomycota core (Dikarya-core; ascomycete and basidiomycete species), (iii) Ascomycota core (Asco-core; hemiascomycete species), (iv) Euascomycete-specific (Euasco-specific; filamentous ascomycete species), (v) \textit{N. crassa} orphan genes (NC-orphans; genes identified only in the \textit{N. crassa} genome), and (vi) Others (gene homologs identified in prokaryotes, nonfungal eukaryotes or basidiomycetes, but not in ascomycete species except for \textit{N. crassa}). We then investigated the relevance of using an evolutionary approach to analyze transcriptome data.

The six groups of LS genes did not show an equal distribution among the six \textit{N. crassa} expression clusters. An enrichment analysis demonstrated an association between expression profiles and phylogenetic distribution of genes (Fig. 4). The most prominent association was between genes within expression cluster 9M, which contains a large percentage of genes classified in the Euk/Prok-core, as well as Dikarya-core genes. Only a small percentage of genes within the \textit{N. crassa} orphan and Euasco-specific lineage groups were identified in this cluster. The Euk/Pro-core and Dikarya-core genes are among the most conserved groups of genes and contain a large number of genes belonging to the “Metabolism (01),” “Energy (02),” and “Protein synthesis (12)” functional categories; these same categories were enriched in the 9M cluster (see page 1 of Table S1 in the supplemental material). In contrast, the other expression clusters (1A, 1B, 3M, 21M, and 27M) contained a relatively larger percentage of \textit{N. crassa} orphan and Euasco-specific genes and a smaller percentage of genes within the Euk/Pro-core and Dikarya-core lineage groups (Fig. 4).

Enrichment analysis revealed that Euasco-specific genes were overrepresented in the clusters 1A, 3M, and 27M (Fig. 4). The 1A cluster contained genes that showed maximal expression at the periphery of the colony, in hyphae that are 0 to 1 h old. These data suggest that filamentous fungus-specific processes are associated with colony extension and tip growth. It is noteworthy that no enrichment of functional categories was detected in the 27M cluster (Table 1).

The 1B cluster of genes showed maximal expression both at the periphery of the colony and at 21 h, suggesting that these genes may be involved in hyphal extension, which occurs both at the periphery of the colony and during aerial hypha formation in the early stages of conidiogenesis. The 21M cluster of genes showed maximal expression in the 21-h-old section of

- **TABLE 4—Continued**

| FunCat category or probe ID\(^a\) | Gene ID | HOPACH profile | MIPS ID | A. niger ortholog | MIPS description\(^b\) | LS group |
|----------------------------------|---------|----------------|---------|-----------------|------------------------|-----------|
|                                  |         | \(N.\) crassa | \(A.\) niger | | | |
| b2n18\_290\_2835             | NCU07999.2 | 1A | 1D | b2n18\_290 | An14g00900 | Probable protein MCD4, required for GPI anchor synthesis | Euk/Pro-core |
| 1nc106\_050\_3237            | NCU08118.2 | 1A | 1D | 1nc106\_050 | An16g04880 | Probable peroxin-1, peroxisomal pex1 | Dikarya-core |
| 4nc675\_170\_494             | NCU08683.2 | 1A | 1D | 4nc675\_170 | An16g04200 | Probable GTP-binding protein rho2 | Asco-core |
| 1nc450\_110\_1454            | NCU09326.2 | 1A | 1D | 1nc450\_110 | An16g07040 | Related to beta-glucosidase | Asco-core |
| b10k17\_080\_966             | NCU09468.2 | 1A | 1D | b10k17\_080 | An01g05650 | Alpha-tubulin B | Euk/Pro-core |
| xnc140\_260\_1459            | NCU09721.2 | 1A | 1D | xnc140\_260 | An16g02490 | Probable adapter-related protein complex 1 | Euk/Pro-core |
| 1nc320\_150\_2980            | NCU09382.2 | 1A | 1D | 1nc320\_150 | An11g10320 | Probable cytoskeleton assembly control protein SLA2 | Dikarya-core |

\(^a\)ID, identifier.

\(^b\)Genes for ergosterol biosynthesis (ERG) were inferred based on homology to genes in \textit{S. cerevisiae}.

\(^c\)Annotation based on the work of Rasmussen and Glass (52).
the colony. Interestingly, in both of these clusters (1B and 21 M), *N. crassa* orphan genes were enriched (Fig. 4). It is intriguing that when *N. crassa* was engaged in conidiogenesis and displaying its species-specific morphological characteristics, a large number of *N. crassa* orphans showed high expression activity.

In summary, the expression timing of genes within LS groups was highly associated with different developmental stages of an *N. crassa* colony. Euasco-specific genes and *N. crassa* orphans are highly active in the colony periphery and during asexual development. In contrast, the relative expression of conserved sets of genes, i.e., Dikarya-core and Euk/Prok-core, was overrepresented in the middle part of the colony. The combination of expression profiling and evolutionary approaches provide testable hypotheses regarding gene function. We have shown that a combination of these approaches will be useful for prioritizing the construction of mutants and has and will aid phenotypic analyses of mutant phenotypes.

**DISCUSSION**

Our time course analysis provides the first global view of gene expression changes during colony development in a filamentous fungus, including extending hyphal tips at the periphery of the colony through to aerial hyphal development and asexual reproduction (conidiation). The examination of colony sections representing distinct developmental stages, Bayesian inference, and robust powerful clustering tools enabled us to delineate and characterize unique patterns of gene expression across a filamentous fungal colony. Enrichment analysis of functional categories highlighted a spatiotemporal differentiation in biological processes occurring in a fungal colony.

**mRNA gradient across the colony.** Hyphae at the periphery of the *N. crassa* colony (0 to 1 h old) showed a spatial pattern of gene expression that was very distinct from that of hyphae in the interior of the colony (6 to 15 h old; 2 to 5 cm behind the periphery of the colony). Genes predicted to be involved in membrane biogenesis, cellular communication/signal transduction, and the cytoskeleton were enriched in the expression cluster associated with the colony periphery. This finding closely parallels biological processes believed to be important for tip growth (32). In contrast, genes predicted to be involved in amino acid, nucleotide, and protein biosynthesis and also in energy generation showed maximal expression in the section of the colony between 6 and 15 h old (2 to 5 cm behind the colony periphery). This set of genes showed low relative expression levels at the colony periphery (0- to 1-h-old hyphae) (Fig. 3B).

Regions of the colony undergoing asexual development (beginning with aerial hypha formation at ~12 h and profuse conidiation observed at 27 h) also displayed expression profiles for a distinct set of genes, mostly of unclassified genes of euascomycete and *N. crassa* orphan lineages. Since an *N. crassa* colony consists of an interconnected, multinucleate syncytium, it is unclear what factors within a colony might promote the variations in expression profiles we observed across a fungal colony. One factor may be differences in nutritional status. Hyphae in the periphery of the colony are exposed to unexplored organic material, whereas its center is surrounded with a substrate that has been utilized (39). Nutritional deprivation is one of the cues for cell differentiation leading to conidio genesis (48).

In *N. crassa*, there are on average ca. 20 septa for every 1 mm of a hyphal segment. These septa have pores that permit nuclei and other, smaller organelles to migrate through as the cytoplasm flows in the direction of growth (18). The cytoplasm in the apical hyphae of the colony migrates at the same rate as tip extension, whereas the mass flow rate in hyphae in internal sections of the colony is ca. 10 times faster than that at the tip (40). In fluid mechanics, the Reynolds number is used to identify and predict different flow regimes, such as laminar or turbulent flows. Lew determined the Reynolds number for cytoplasmic flow in *N. crassa* hyphae to be ~10^−4 (40). With a Reynolds number as low as 10^−4, particles and molecules can mix only by diffusion, and thus, mRNAs produced in one cell will not mix efficiently with mRNAs produced in neighboring cells. Previously it was shown that the excision of 1 cm or more behind the periphery of a colony does not affect the growth rate of the leading hyphae (57, 74, 83); this region is defined as the “peripheral growth zone.” Endoplasmic reticulum, Golgi apparatus, polysomes, and mitochondria are abundant in subapical regions of a colony (within the 0- to 1-h-old section) (31, 71). Thus, transport from regions of a hypha further from the tip than 1 cm is not essential for maintaining the growth rate. However, expression of genes encoding proteins involved in protein biosynthesis and energy production showed nadir expression in the 0- to 1-h-old section of the colony (3 mm wide). The relative expression level of these same genes increased until maximal expression occurred 2 to 5 cm behind the colony periphery. Thus, our mRNA profiling data imply an existence of an active transportation system for proteins involved in protein synthesis, energy metabolism, and amino acid biosynthesis from the midsection of the colony toward the periphery, where maximal growth is occurring. For example, microtubules and their associated motor proteins, the dynein and kinesin family members, are important for long-distance transport of mitochondria and vesicles and for nuclear positioning (80). An alternative possibility is that even though mRNA for proteins involved in protein synthesis/energy metabolism/amino acid biosynthesis is more abundant in the midsection of the colony, differences in translational efficiency may result in a more uniform distribution of these proteins across a fungal hypha. Future studies assessing protein composition and translational capacity across a fungal colony will provide informative data to distinguish these two possibilities.

**Transcriptional profiles of the colony periphery reflect polarity processes.** Hyphal extension in filamentous fungi is driven by cytoskeleton-based polar exocytosis. Based on the “vesicle supply center” model, secretory vesicles produced in the Golgi apparatus are delivered to the Spitzenkörper via kinesin-mediated transportation along microtubules (32). Exocytic vesicles are then released from the Spitzenkörper and delivered to the tip via myosin-mediated transportation along F-actin. Rapid growth of *N. crassa* requires ~38,000 vesicles fusing with the tip per minute (16, 66). Due to its extremely high growth rate and its highly elongated form, Seiler and Plamann advocated the use of *N. crassa* as a model for the analysis of fundamental mechanisms underlying cellular polarity (61). We identified a conserved set of genes required for polar growth that showed maximal activity at the periphery of
the colonies in both *A. niger* and *N. crassa*. In 6- to 15-h-old hyphae in *N. crassa*, genes for amino acid, nucleotide, and protein biosynthesis and also for energy generation showed maximal expression. In *A. niger*, many of these gene orthologs were also coexpressed in 0- to 2-day-old sections of the colony. For *N. crassa*, the high growth rate and ability to section the colony on a fine time scale enabled us to discern patterns of gene expression across a fungal colony that would be technically difficult with other slow-growing fungal species.

Our research further emphasized the advantages of using *N. crassa* for cellular events associated with tip growth. The hyphal tip is the primary location for growth and morphogenesis in filamentous fungi. Because of this, mutations in genes showing maximal expression at the colony periphery may be expected to result in severe growth phenotypes. Indeed, our mRNA profiling demonstrated that for both UV random mutagenesis and gene deletion, mutations in genes that showed maximal expression at the periphery of a colony were more likely to result in a mutant growth phenotype. Out of the 45 genes whose mutations were responsible for the temperature-sensitive growth phenotypes (61), 43 of the corresponding gene models are found in the MIPS database. Of these genes, 41 encode very conserved proteins; 16, 20, and 5 genes belong to the LS groups Euk/Prok-core, Dikarya-core, and Ascomycota-core, respectively. The remaining two genes belong to the Euasco-specific group, and none belongs to the *N. crassa* orphan group. Only 4 out of the 43 cloned genes (9%) correspond to “Unclassified proteins,” despite the fact that 56% of genes in the *N. crassa* genome code for “Unclassified proteins.”

We predict that mutations in many genes that showed maximal expression at the hyphal tip may lead to growth and polarity phenotypes; transcriptional profiling data can thus be used to prioritize phenotypic characterization of gene deletion mutants (17, 19).

**Interpretation of colony development according to lineage specificity of genes.** The phylogenetic distribution of a gene has been suggested to be of biological importance (38). Genes with the same phylogenetic distribution may have linked functions (1, 45, 50). If this is the case, then a correlation between phylogenetic distribution and fungal development can be evaluated. We identified an enrichment of Dikarya-core and Euk/Prok-core genes in the 9M cluster, which was also associated with the enrichment of FunCat categories of “Metabolism,” “Energy,” and “Protein synthesis.” The expression of Euasco-specific genes showed an overrepresentation in hyphae at the colony periphery and in hyphae undergoing asexual development (conidiation). Because more than 76% of the genes in the Euasco-specific LS group are unclassified genes, the contribution of any particular functional category in this LS group is not clear. A comparative genomics study with *Saccharomyces* and *Aspergillus* genera showed that functions of highly lineage-specific genes were poorly characterized or simply unknown (14). Many lineage-specific genes may be involved in niche adaptation, such as conspecific or heterospecific interactions in biocenosis; mutations may not result in perceptible phenotypic changes in axenic culture.

The expression of *N. crassa* orphan genes was also overrepresented at the colony periphery and during conidiogenesis. Molecular functions of *N. crassa* orphans are mostly unknown. Cal and associates showed an increase in nonsynonymous nucleotide substitution rates in genes with higher LS in ascomycete fungi (14) and in primates (James Cai, personal communication). It is likely that *N. crassa* orphans are among the fastest-evolving genes, so that their homologs are no longer identifiable in genomes of closely related fungal species. In mammals, tissue-specific genes evolve more rapidly than widely expressed genes (20). Genetic mutations in genes expressed early in development or in many different tissues (20) are expected to exhibit more pleiotropic effects than those expressed late in development or showing tissue-specific expression. If *N. crassa* orphan genes evolve fastest among all of the LS groups, the enrichment of *N. crassa* orphans during conidiogenesis may be analogous to the general trend of fast evolutionary rates observed in tissue-specific genes or genes expressed late in development. Interestingly, we determined that *N. crassa* orphan genes are clustered near telomerases (unpublished data), a region of the chromosome that has been associated with fast-evolving genes in other species (22, 23, 59, 79). At present we do not have experimental data to test whether *N. crassa* orphans expressed during conidiation are under positive selection or relaxed functional constraint. The ongoing genome projects with several *N. crassa* sister species will enable us to investigate the selective force driving the evolution of *N. crassa* orphan genes.

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