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Use of expressed sequence tag analysis and cDNA microarrays of the filamentous fungus *Aspergillus nidulans*

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Abstract

The use of microarrays in the analysis of gene expression is becoming widespread for many organisms, including yeast. However, although the genomes of a number of filamentous fungi have been fully or partially sequenced, microarray analysis is still in its infancy in these organisms. Here, we describe the construction and validation of microarrays for the fungus *Aspergillus nidulans* using PCR products from a 4092 EST conidial germination library. An experiment was designed to validate these arrays by monitoring the expression profiles of known genes following the addition of 1% (w/v) glucose to wild-type *A. nidulans* cultures grown to mid-exponential phase in Vogel's minimal medium with ethanol as the sole carbon source. The profiles of genes showing statistically significant differential expression following the glucose up-shift are presented and an assessment of the quality and reproducibility of the *A. nidulans* arrays discussed.

Keywords: *Aspergillus nidulans*; Microarray; EST; Alcohol; Ethanol; Glucose repression; *creA*

1. Introduction

Functional genomics reverses the normal course of genetics research, by starting with the genes and attempting to define their function (Oliver, 1996). In this approach, genes are defined by the bioinformatic analysis of genomes that have been completely (or extensively) sequenced, and a wide variety of high-throughput technologies are used to investigate gene function at the levels of transcriptome, proteome, and metabolome (Oliver, 2000). The ultimate aim of functional genomics is to provide an integrative or holistic view of the workings of living cells, and this 'systems' approach to biology is being pursued mainly with model organisms, such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, and *Caenorhabditis elegans*. A truly integrative view of gene action and interaction in these model species is some way off, and it might be contended that classical approaches are perfectly adequate to uncover the functions of individual genes discovered by genome sequencing. Whatever the merits of this argument, the same cannot be said of less-developed experimental organisms, where there must be considerable hope that functional genomic approaches will significantly accelerate the research effort.

The yeasts *S. cerevisiae* and *Schizosaccharomyces pombe* are members of the ascomycete fungi, but most members of this group have a filamentous, rather than a unicellular, growth habit. The genus *Aspergillus* comprises a particularly important group of filamentous ascomycete species. It includes members that have important roles in nature via the degradation of plant

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polysaccharides (de Vries and Visser, 2001), others that are involved in food spoilage (Rustom, 1997) and food production, as well as the major filamentous fungal pathogen of humans—*Aspergillus fumigatus* (Denning et al., 2002). Moreover, Aspergilli are important industrial microorganisms for the large-scale production of both homologous and heterologous enzymes (Archer, 2000; Conesa et al., 2001; Finkelstein et al., 1989) and pharmacologically active compounds (Askenazi et al., 2003; Manzoni and Rollini, 2002).

Unlike most of the Aspergilli, *Aspergillus nidulans* exhibits a sexual cycle and it has become an important model for genetic studies of the filamentous fungi (Martinelli, 1994). Despite the importance of *A. nidulans*, the cohort of researchers involved in its study is far smaller than that for, say, *S. cerevisiae*. Thus, the application of functional genomic analyses to *A. nidulans* should have a significant impact on the progress of research with this species. Amongst the functional genomic technologies, it is the use of hybridization-array analysis for the global study of gene expression at the level of mRNA synthesis that has had the greatest and most immediate impact (Aoyagi et al., 2003; Puthoff et al., 2003; Wurmbach et al., 2002). In this paper, we report the establishment of hybridization-array analysis for *A. nidulans*.

Two main problems have been encountered in achieving this. First, a complete annotated genome sequence of *A. nidulans* is only just about to become available. We have, therefore, fabricated microarrays using 3752 PCR products from expressed sequence tags (EST) produced from cDNA libraries (see Section 2) made from asexually developing (conidiating) cultures of *A. nidulans* and 340 PCR products of *Aspergillus* sequences deposited in GenBank and have used sequence comparisons, both to the emerging *A. nidulans* genome sequences and to those of other ascomycete fungi, to annotate the redundant set of genes represented in these arrays.

The second major problem relates to the filamentous growth habit of *A. nidulans*. The mycelium tends to pellet when grown on glucose as the principal carbon source (Pirt, 1967). Since hyphae at the centre of the pellet will suffer both nutrient and oxygen deprivation compared to hyphae at the periphery (Metz and Kossen, 1977), this means that cultures will not be physiologically homogeneous. This will, at the very least, complicate the transcriptome analysis and may perhaps negate its validity altogether. However, when *A. nidulans* is grown on ethanol, rather than glucose, it grows in a dispersed (and so, physiologically homogeneous) manner (this study). What is more, it continues to grow in this way when glucose is added to the medium. For this reason, we have chosen an ethanol-to-glucose up-shift experiment to test the utility and validity of our arrays. A further advantage is that this particular transition results in a physiological change that has been relatively well characterized in *A. nidulans* and is due to the expression of the negatively acting global repressor, CreA (Ruijter and Visser, 1997). Finally, there are data available in the literature for equivalent transitions in both yeast (DeRisi et al., 1997) and filamentous fungi (Chambergo et al., 2002) and these provide a set of expected results against which the array data for *A. nidulans* may be compared.

2. Materials and methods

2.1. Strain storage and spore production

Wild-type *A. nidulans* (FGSC A1004) spores were stored in 20% (v/v) glycerol at −80 °C until required. Spores were harvested from 7- to 10-day-old MEA plates (3% (w/v) malt extract, 0.3% (w/v) peptone, 2% (w/v) agar, and 2% (w/v) glucose) with 5 ml 0.01% (v/v) Tween 80 by gently agitating the surface with a glass spreader. Spores were filtered through two layers of lens tissue (Whatman), centrifuged at 3600*g*, resuspended in sterile water to give a final concentration of 10^9 spores/ml and stored at 4 °C until required.

2.2. Growth and culture conditions

The *A. nidulans* strain was grown to mid-exponential phase at 37 °C in 50 ml Vogel's minimal medium containing 10^7 spores/ml (Vogel, 1956) containing 1% (v/v) ethanol as sole carbon source. Ethanol was chosen as it does not cause carbon catabolite repression (Arst and Cove, 1973) and had previously been shown to produce dispersed filamentous growth in liquid culture (results not shown). The mid-exponential phase culture was used to inoculate a 2.1 L fermentation vessel (Braun Biostat M) containing Vogel's medium with 1% (v/v) ethanol and the culture maintained at 30 °C with constant stirring (1000 rpm) and aeration (2.1 L/min). Growth was monitored by measuring CO_2 production on-line. Samples (50 ml) were removed from the fermenter immediately before and 1, 2, and 4 h after the addition of glucose (to a final concentration of 1% w/v) directly to the vessel. Samples were rapidly filtered under vacuum, flash-frozen in liquid nitrogen, ground to a fine powder in an RNase-treated mortar and pestle, and the RNA extracted immediately as described below.

2.3. RNA extraction

Ground and frozen mycelium was transferred to 1.5 ml Eppendorf tubes, dissolved in 1 ml of TriZol reagent (Sigma–Aldrich, UK), and treated as described in Hayes et al. (2002). Total RNA extracted using this procedure was used to synthesize labelled targets for hybridization to microarrays.

2.4. Microarray feature preparation

Polymerase chain reactions (PCRs) were performed in 96-well plates containing 100 μl of reaction solution with 10 pmol/μl of 5′ amino-modified array primer (hybridized to the vector-based T7-recognizing motif) and 10 pmol/μl of a gene-specific primer. For each unique EST or *A. nidulans* ORF deposited in GenBank, a gene-specific 18-mer oligonucleotide was synthesized and used to PCR-amplify specific DNA fragments. The template used in all PCR amplifications was 1 μg of a double-stranded plasmid cDNA library constructed from mRNA extracted from asexually developing tissues and a single-stranded λZAP library (lodged at the Fungal Genetics Stock Centre, and constructed originally by Dr. R. Aramayo, Texas A&M University).

PCRs were initiated with DNA denaturation (3 min at 96 °C) followed by annealing (30 s at 50 °C) and extension (1 min at 72 °C) steps carried out for 34 cycles. Aliquots (15 μl) of all PCR amplification products were analysed by 2% (w/v) agarose gel electrophoresis after ethidium bromide staining and only products that yielded a single band were stored for microarray printing.

2.5. Fabrication of microarrays

Microarrays containing 3572 spotted PCR products of ESTs of approximately 500 bp isolated from conidial libraries and 340 PCR products of *Aspergillus* sequences deposited in GenBank were immobilized onto coated glass microscope slides (Corning CMT-GAPS II) using a Biorobotics Total Array System robot. Probes were spotted onto the glass slides using a 4×4 print head and each of the corresponding 16 print tip groups was laid out in duplicate sets of 17×16 grids, 200 μm apart.

2.6. Labelled target synthesis

Fluorescently labelled targets were synthesized using the method of Hegde et al. (2000) with two modifications. First, the amount of total RNA used was increased to 60 μg and, second, an anchored oligonucleotide (oligo $dT_{15}VN$, where V is any nucleotide except thymidine and N is any nucleotide) was used as a primer for the first-strand syntheses. Incorporation of fluorescent Cy5-labelled nucleotides into the cDNA target was visualized by running 1 μl of the 50 μl eluted from the GFX column on a 1.2% (w/v) agarose gel and scanning the gel with a STORM 860 phosphorimager (Molecular Dynamics, USA); Cy3 is not visible using this scanner.

2.7. Hybridization to microarrays

The microarrays, described above, were used with fluorescently labelled targets. The two labelled targets, each in 10 μl water, were combined and added to 10 μl hybridization buffer containing 50% (v/v) formamide, 10× SSC, 0.2% SDS and heated to 100 °C for 3 min. Hybridization was then carried out as described in Hayes et al. (2002).

2.8. Image analysis

Images of the microarrays were acquired using a GenePix 4000A microarray scanner (Axon Instruments, USA) and the spots quantified using GenePix Pro 3.0 software.

3. Data analysis

3.1. Normalization

The raw data consisted of 20 hybridizations (10 dye-flip pairs) on duplicate printed slides from the same print batch. Three biological replicates were used corresponding to three different fermentations, distributed over all the time points studied (1, 2, and 4 h). Given these multiple data sets, we wished to obtain an accurate indication of the proportional changes of gene expression at the different time points following glucose addition. For each hybridization we only used those spot measurements that had not been flagged by the scanning software and for which positive intensities, from each channel, were obtained following background correction. Background-corrected intensities were calculated as the difference between the median foreground and median background intensities, as estimated by the GenePix Pro software. The raw log.-ratios contain a number of biases, in particular intensity-dependent and spatial biases. Analysis of variance (ANOVA) (Kerr et al., 2000) was applied to the unnormalized log.-ratios (from all slides and all time points), to assess the degree of variation due to a number of factors. Between-replicate slides was the greatest source of variation (after correcting for the number of degrees of freedom), accounting for approximately 76% of the total variation, whilst within-replicate slide variation (including duplicate printing) accounted for approximately 13%. We did not use an explicit ANOVA model to remove these systematic errors and normalize the log.-ratios. Instead, for each data set, we applied loess smoothing of the raw log.-ratios from each print-tip group, following Yang et al. (2002). Loess-corrected log.-ratios from different print-tip groups were combined using a location and scale transformation (Yang et al., 2002). Finally, we explicitly set the median log.-ratio to zero. Once each hybridization had been loess-corrected, then for a given time point (1, 2, and 4 h), a final normalized log.-ratio for probe was obtained by averaging over all the

hybridizations from that single time point. Following Tseng et al. (2001), we monitored the reproducibility and quality of data by calculating the coefficient of variation, CV (across all the replicate measurements) of the normalized ratio of each probe, for each time point. For the 1 h samples the average (across probes) CV was 0.22 (standard deviation = 0.16), whilst for 2 h samples average CV = 0.21 (std. dev. = 0.16) and for 4 h samples average CV = 0.22 (std. dev. = 0.17). This indicates that there is a consistent typical "signal-to-noise" ratio of approximately 5:1 for a single normalized hybridization. Similarly an average (over technical replicates) normalized log.-ratio was obtained for each probe and particular combination of time point and fermentation. A coefficient of variation of the normalized ratio, for each probe and time point, was then calculated over the different fermentations. The average (over probes and time points) of this fermentation coefficient of variation was 0.18 (std. dev. = 0.18), indicating good reproducibility of the normalized data between fermentations.

Within a single time point and for each replicate data set i, a residual error, ε_{ij}, was then calculated as the difference between the final normalized log.-ratio for probe j and the loess-corrected log.-ratio for probe j from replicate i. Visual inspection of the residuals did not give any cause to reject an assumption of homoskedascity. Therefore, we have taken the residual error, ε_{ij}, to be dominated by non-systematic experimental error that is characterized only by the particular replicate i. Significance levels (p values) of the final normalized log.-ratios for each probe were calculated by re-sampling suitably scaled values from the residuals of each hybridization (Kerr et al., 2000; Wu, 1986). In testing the normalized log.-ratio of a probe, 5000 bootstrap data sets were generated by this re-sampling approach, taking care to note which specific hybridizations were used in constructing the normalized log.-ratio for that probe. This significance-testing procedure was repeated for each probe. In this way, we avoided making parametric assumptions about the mathematical shape of the distribution of the residual errors—in contrast, for example, to using a t test to determine significance level. From this, we can identify those genes that are genuinely differentially expressed between the two labelled populations of mRNAs at each time point. Initially, we identified genes as being significantly differentially expressed if they had a $p < 0.01$. In addition, by estimating the q values of each normalized log.-ratio (the probability of a log.-ratio being a false positive, given it appeared significantly differentially expressed), we can assess the false discovery rate. We have calculated q values by the method of Storey (2002, 2003) by which we found that those genes which had $p < 0.01$ corresponded to a q value <0.034.

4. Results and discussion

In this section, we will first describe the characteristics of the arrays that we have fabricated in order to carry out transcriptome analyses for *Aspergillus*. Next, we will describe the glucose up-shift experiment that we have used as a test object to evaluate the utility and validity of the arrays. We will then attempt to predict the likely outcome of the transcriptome experiment based on data obtained by others using more conventional gene expression analyses. Finally, the results of our microarray analysis will be presented and discussed, both in terms of the use of the technology and of the biology of *A. nidulans*. Raw data are available on the COGEME (Consortium for Functional Genomics of Microbial Eukaryotes) web site (http://www.cogeme.man.ac.uk).

4.1. Attributes of the array

A picture of a typical hybridization to our *A. nidulans* EST arrays is shown in Fig. 1. The array consists of 4092 individual spots arrayed in 16 blocks of 16×17 spots. However, there are 520 duplicated sequences and many genes are represented by more than one non-overlapping sequence. This level of redundancy reduces the potential coverage of the array, but is a useful feature in the evaluation of the reproducibility of the arrays and of the techniques used. The redundancy comprises both duplicated sequences and multiple sequences representing different sections of the same genes as identified by BLAST searches.

Comparisons of the expression profiles of different EST sequences that have sequence similarity to alcohol dehydrogenase I from different species are shown in Fig. 2. Only the sequences with BLAST matches to *A. nidulans* alcohol dehydrogenase, *alcA* (Fig. 2) have convincing and reproducible expression profiles. Sequences on the array with BLAST matches to alcohol dehydrogenases of non-*Aspergillus* species had expression profiles that were different to those of the *A. nidulans* sequences in that they showed no down-regulation after addition of glucose to the culture medium. Sequence similarities to alcohol dehydrogenase I of non-*Aspergillus* species suggested by BLAST matching are therefore unreliable and it is concluded that these sequences do not represent *alcA* and any nucleotide or amino-acid similarities are due to chance events and these sequences may therefore represent other *A. nidulans* genes that have not been identified previously or, indeed, other alcohol dehydrogenases genes in *Aspergillus* that are not regulated (Jones et al., 2001) during carbon source up-shift.

In general, spots representing the same sequences show very low variation and the differences in the

expression profiles are not significant. For example, see spots 2240 and 2400 or 871 and 1065, representing *A. nidulans alcA* (Fig. 2). Nevertheless, some sequences on the array that appear to represent different segments of the same gene show some variation in expression profiles. This may be because they are members of a gene family, for example, alcohol dehydrogenase I and III, chitin synthase a and b (see Table 2). Differences in expression profiles could also reflect mRNA processing phenomena, such as differential splicing.

Table 1 summarizes the identities of all the ESTs on the array. BLAST searches identified 1922 sequences on the array with possible identities. Of the top hits, 977 are fungal sequences, 614 represent *Aspergillus* sequences, and 292 represent known *A. nidulans* genes. Release of a putative gene-set (ORF list) predicted by automated annotation by the Whitehead Institute allowed determination of which ORFs are represented by the respective ESTs using BLASTn matching to predicted cDNA sequences. Fig. 3B illustrates those that are identified as *Aspergillus* genes and which are identified as orthologues of genes of fungal or other origin. The majority of the ESTs have no identity as yet, and therefore represent a rich resource for the discovery of new *Aspergillus* genes. Fig. 3A illustrates the annotations of those ESTs identified as being derived from previously characterized *Aspergillus* genes. Table 4 lists the known *A. nidulans* genes included on the array.

4.2. The test experiment

In order to evaluate the microarrays, we performed a batch fermentation of *A. nidulans* A1004 on Vogel's minimal medium with 1% (v/v) ethanol as carbon source. When the culture had reached early exponential phase, glucose was added to a final concentration of 1% (w/v). This glucose up-shift experiment is deceptively simple. However, in terms of the genome-wide change in the organism's transcription profile, it is rather complex, and the response can be predicted to contain a number of different components. First, an increase in the growth rate of the culture can be expected as it switches from using ethanol to using glucose as its primary source of carbon. Second, it can be predicted that the expression of genes subject to carbon catabolite repression will be shut down on transfer to glucose, the archetypical repressive carbon source. Finally, with an alcohol as the carbon source, the fungus would need to generate the glucose required for anabolism internally, thus gluconeogenic pathways (including the glyoxylate cycle) should be switched on whilst growing on ethanol and then be repressed upon transfer to glucose.

4.3. The impact of growth rate on gene expression

The first predicted component of the response to glucose up-shift, that of a growth rate increase, was easily demonstrated. The growth rate of the culture increased slightly 2 h after the addition of the glucose, as shown in Fig. 4 (maximum growth rate, $\mu_{max} = 0.28\,h^{-1}$ on ethanol and $0.59\,h^{-1}$ after glucose addition). This growth rate increase was reflected, at the level of the transcriptome, by an increase in the expression of genes encoding ribosomal proteins (see Table 3). It has been amply demonstrated, in *S. cerevisiae*, that the transcription of genes encoding ribosomal proteins (as well as components of the transcriptional apparatus) is directly correlated with growth rate (Hayes et al., 2002; Lim et al., 2003). The change in growth rate also had an effect on the expression levels of some 'house-keeping' genes such as β-tubulin, which showed a small (1.56-fold), but significant, decrease in expression after 2 h and actin, which also showed a small (1.41-fold) decrease in expression. A statistically significant decrease in the numbers of genes expressed during increased growth rate has been observed in *S. cerevisiae* transcriptome experiments. In these experiments, actin has been seen to exhibit a slight down-regulation, but tubulin does not show a similar response (L. Zeef, D.C.J. Gardner, A. Hayes, S.G. Oliver, unpublished results.) The stress response gene Hsp1 also showed a significant down-regulation after 2 h (average of 1.33-fold), which probably represents an adaptation of the culture to growth on glucose.

4.4. Glucose repression effects

In *A. nidulans*, the repressor protein CreA plays a major role in carbon catabolite repression, of which glucose repression is a specific case (Ronne, 1995; Ruijter and Visser, 1997). In only a limited number of systems has it been clearly shown that the relevant genes are repressed, for example, the ethanol regulon (Mathieu and Felenbok, 1994) and proline utilization gene cluster (Cubero and Scazzocchio, 1994; Sophianopoulou et al., 1993). Of the systems regulated by carbon repression, the main group comprises genes encoding enzymes involved in the catabolism of less favourable carbon sources, e.g., enzymes involved in the degradation of polysaccharides such as cellulose, pectin, xylan, and arabin. A second group includes genes encoding gluconeogenic and glyoxylate cycle enzymes. The gluconeogenic enzymes may not be glucose-repressed (Ruijter and Visser, 1997), and Kelly and Hynes (1981) have reported that phosphoenolpyruvate carboxykinase is only weakly regulated by carbon repression. A third group of genes subject to carbon catabolite repression is related to secondary metabolism and includes penicillin production by *ipn*A, encoding isopenicillin *N*-synthetase

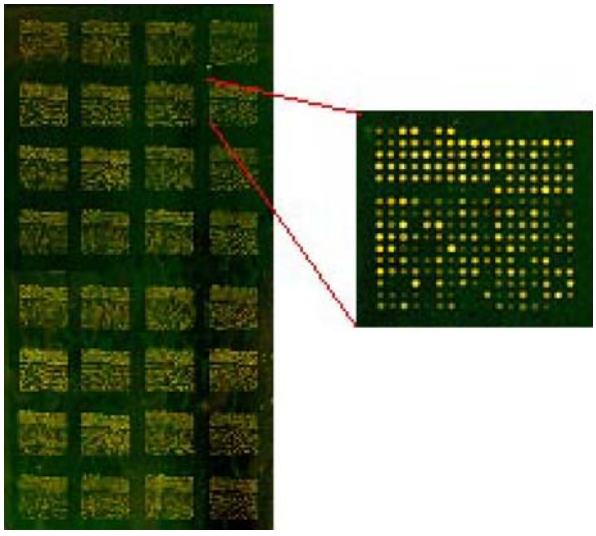


Fig. 1. A typical hybridization image of a microarray showing duplicate sets of 4092 PCR products spotted in 17×16 grids.

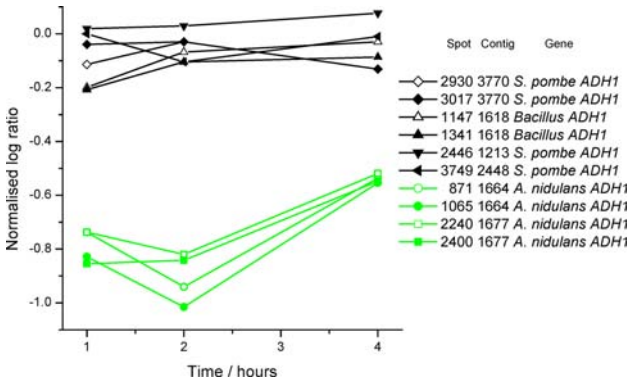


Fig. 2. Expression profiles (after the addition of glucose) of ESTs with sequence similarity to alcohol dehydrogenase I from various species as determined by BLASTx matches to protein sequences in GenBank. Multiple spots are represented by the same EST (distinguished by contig number).

(Brakhage, 1998). In *S. cerevisiae*, enzymes involved in the citric acid cycle and the respiratory chain are also repressed by glucose but, in *Aspergillus* (an obligate aerobe), this would not be expected and Raitt et al. (1994) have shown that cytochrome *c* synthesis is not repressed by glucose in *A. nidulans*.

The addition of glucose, a favoured carbon source to the culture vessel should induce CreA (the negative-acting regulatory protein mediating carbon catabolite repression) that acts directly on *alcA* (encoding alcohol dehydrogenase) and indirectly via the regulatory *aldR* gene on *aldA* (aldehyde dehydrogenase) (Flipphi et al., 2002, 2003). There are multiple copies of alcohol dehydrogenase coding sequences represented on the microarray (see Table 1), which show significant down-regulation 1 and 2 h after addition of glucose (e.g., an average 2.48-fold for *A. nidulans adhA* at 2 h). The

Table 1
BLAST searches identified 1922 sequences on the array with possible identities (2170 produced no matches)

No. of ESTs	3752	
No. of PCR products	340	
Total No.of Sequences	4092	
Duplicated sequences	520	13%
Unique sequences	3572	87%
Sequences representing *A. nidulans* ORFs	3304	81%
Unique *A. nidulans* ORFs represented	2080	(22% of predicted 9541 ORFs)

Of the top hits, 977 are fungal sequences, 614 represent *Aspergillus* sequences, and 292 represent known (isolated and sequenced) *A. nidulans* genes.

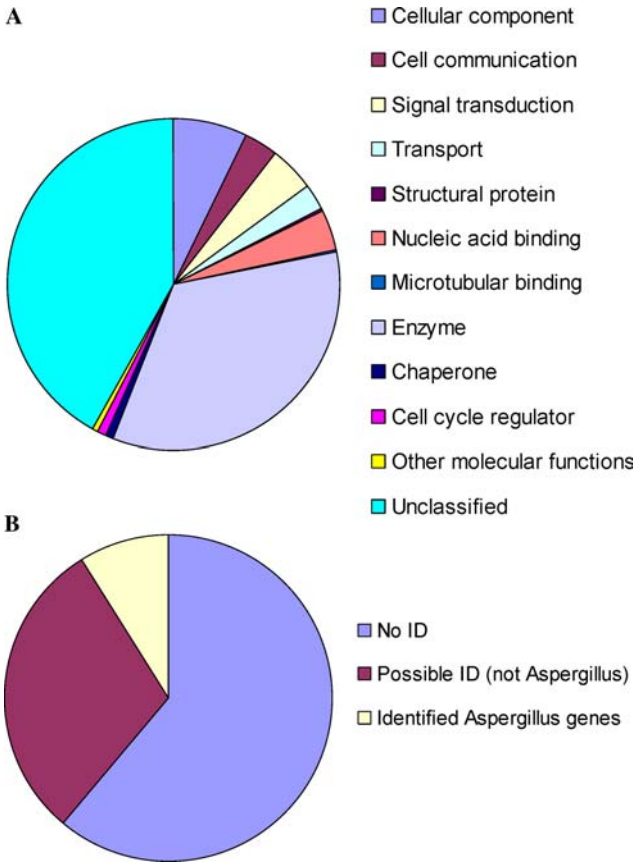
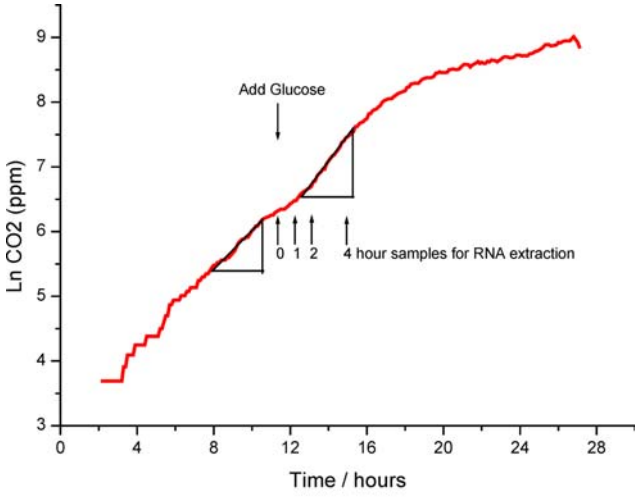


Fig. 3. Identities and annotations of *Aspergillus* sequences on the array. (A) Identified *Aspergillus* sequences. (B) All ESTs.

transcription of the aldehyde dehydrogenase gene, *aldA*, also showed a significant down-regulation but at a lower level, 1.69-fold.

As mentioned above, it was expected that *creA* itself would be induced following glucose addition. However, a 1.47-fold down-regulation of *creA* was observed 2 h after glucose addition. This has been reported previously (Strauss et al., 1999) and is typical of carbon catabolite repression, which requires a functional CreA recogni-



Experiment	Control fermentations		Up-shift	
Carbon source	Ethanol only	Glucose only	Ethanol (before)	Glucose (after)
μ_{max}	0.25	0.54	0.28*	0.59*

* averages of 3 repeats

Fig. 4. Changes in growth rate due to glucose up-shift and times of sampling for microarray analysis. Samples were taken before addition of glucose (time 0) and after 1, 2, and 4 h for RNA preparation and at time 0 and 2 h for growth rate measurements. The average specific growth rate (μ) changed significantly (two sample *t* test, $p = 0.0068$) as a result of the glucose up-shift.

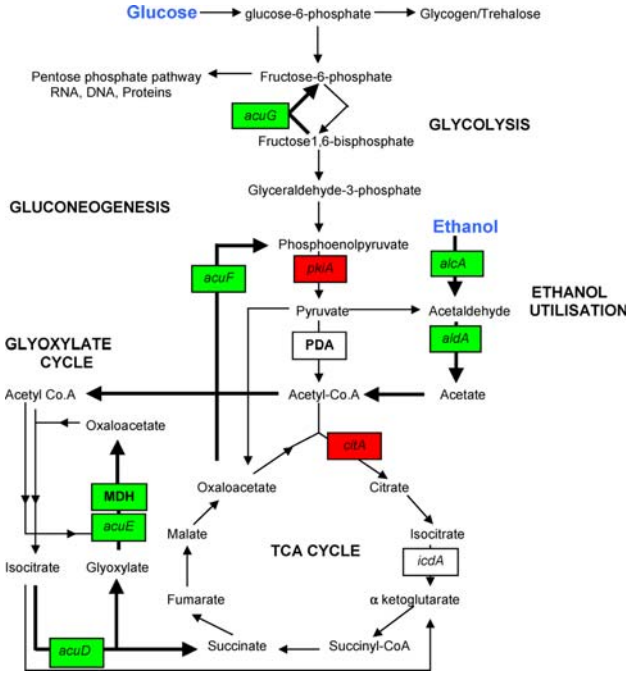


Fig. 5. Pathway for glucose and ethanol metabolism, showing differential expression levels in enzymes of the glycolysis, gluconeogenesis, glyoxylate, and TCA cycles due to a glucose up-shift. Boxes show *A. nidulans* enzymes represented on the microarray. Red and green represents genes significantly up/down regulated, respectively. Dark arrows represent ethanol metabolism, which is strongly repressed after the glucose up shift. Pyruvate dehydrogenase (PDH) and malate dehydrogenase (MDH) have not previously been sequenced for *A. nidulans*.

tion site in the *creA* promoter (and thus involves autoregulation) and the formation of glucose-6-phosphate. Strauss et al. (1999) reported that glucose repression in *A. nidulans* involves both transcriptional and post-transcriptional regulation of *creA*. They found that carbon sources such as ethanol, which are carbon catabolite derepressing, lead to a continuous slow accumulation of *creA* mRNA, which reached levels of similar abundance to those present at the peak produced 90 min after the addition of glucose. Thus, in our fermentation, the culture growing on ethanol would already have high levels of *creA* transcripts and the addition of glucose would not be expected to elicit a further increase in *creA* mRNA levels. Moreover, as *creA* is negatively autoregulated, this could lead to a reduced steady-state level of the *creA* transcript, as was observed. One interesting observation is the down-regulation of *scon*C (2.25-fold at 2 h), a gene involved in sulphur metabolite repression and which has a CreA-binding site (Piotrowska et al., 2000).

The *facB* gene of *A. nidulans* encodes the major regulator of genes involved in acetate utilization and is required for acetate induction of acetamidase (*amdS*) and of the acetate utilization enzymes acetyl-CoA synthetase (*facA*), isocitrate lyase (*acuD*), and malate synthase (*acuE*) (Todd et al., 1998). Growth in ethanol would lead to an induction of *facB* and subsequent up-regulation of these genes. The addition of glucose to the culture would therefore lead to the observed down-regulation of *facB* (1.45-fold) and this, in turn, would lead to the observed down-regulation of *facA* (1.64-fold after 2 h), *acuD* (5.46-fold after 4 h), and *acuE* (6.54-fold after 4 h).

4.5. The response of genes encoding enzymes involved in the glyoxylate cycle and gluconeogenesis

The glyoxylate cycle is a modified form of the TCA cycle, which enables plants, animals, and microorganisms to utilize acetate formed from ethanol for the biosynthesis of carbohydrates. Fig. 5 illustrates the key enzymes in this pathway, isocitrate lyase (encoded by *acuD*) and malate synthase (specified by *acuE*), which produce oxaloacetate and succinate, respectively. These intermediates are subsequently used in gluconeogenesis. Addition of glucose to the culture vessel resulted in a marked down-regulation of the expression of these genes (5.5-fold for *acuD* and 6.5-fold for *acuE*). The gene for fructose 1,6-bisphosphatase (*acuG*), a key enzyme in gluconeogenesis as well as a control point for glycolysis (Yin et al., 2003), also showed a significant down-regulation (2.1-fold). Genes specifying enzymes for aconitase and malate dehydrogenase also showed down-regulation following glucose addition (3.15-fold and an average of 2.04-fold, respectively).

Table 2
Table of fold changes (following addition of glucose) of genes encoding enzymes in key pathways

Spot No.	1 h	2 h	4 h	EST/PCR product	Gene
Glycolysis pathway					
3789	1.07	1.11	1.13	contig1999Oct161435_1635	6-Phosphofructokinase
1941	−1.01	−1.01	1.03	contig1999Oct161435_3630	Pyruvate kinase (pkiA)
3074	*1.48	*1.56	*1.32	contig1999Oct161435_1131	Pyruvate kinase (pkiA)
3489	−1.02	1.10	1.08	contig1999Oct161435_3638	Pyruvate kinase (pkiA)
277	*2.02	*1.60	*2.80	PCR	Pyruvate kinase (pkiA)
655	*1.65	*1.43	*1.75	PCR	Pyruvate kinase (pkiA)
1857	*1.60	*1.51	*1.99	contig1999Oct161435_3630	Pyruvate kinase (pkiA)
2093	1.08	−1.04	1.06	contig1999Oct161435_4018	Pyruvate dehydrogenase
191	1.08	1.04	1.12	contig1999Oct161435_2803	Pyruvate dehydrogenase
TCA cycle					
320	−1.11	1.02	*1.31	contig1999Oct161435_2342	Citrate synthase mitochondrial precursor (citA)
985	−1.07	1.06	*1.28	contig1999Oct161435_2343	Citrate synthase mitochondrial precursor (citA)
2925	1.00	1.04	−1.00	contig1999Oct161435_3910	Isocitrate dehydrogenase (icdA)
3336	1.02	1.11	−1.01	contig1999Oct161435_2929	Isocitrate dehydrogenase (icdA)
Gluconeogenesis pathway					
4301	−1.12	−1.19	*−1.39	contig1999Oct161435_4099	Phosphoenolpyruvate carboxykinase (acuF)
4335	*−2.92	*−2.63	*−3.49	contig1999Oct161435_1534	Phosphoenolpyruvate carboxykinase (acuF)
2575	*−1.73	*−2.02	*−2.14	contig1999Oct161435_3030	Fructose-16-bisphosphatase (acuG)
Glyoxylate cycle					
2713	*−1.80	*−1.91	*−2.22	contig1999Oct161435_1644	Malate dehydrogenase (glyoxylate)
2519	*−1.56	*−1.66	*−1.86	contig1999Oct161435_1644	Malate dehydrogenase (glyoxylate)
3371	*−3.37	*−4.20	*−6.54	PCR	Malate synthase (glyoxylate—acuE)
3394	−1.02	1.02	−1.07	contig1999Oct161435_958	Isocitrate lyase (acuD)
1361	*−2.84	*−3.87	*−5.46	PCR	Isocitrate lyase (acuD)
2551	−1.08	1.05	*1.32	contig1999Oct161435_930	Isocitrate lyase (acuD)
Ethanol utilization pathway					
371	*−2.09	*−2.56	−1.71	contig1999Oct161435_1664	Alcohol dehydrogenase I (alcA)
1065	*−2.29	*−2.76	−1.74	contig1999Oct161435_1664	Alcohol dehydrogenase I (alcA)
1485	1.07	1.08	−1.01	contig1999Oct161435_742	Alcohol dehydrogenase III (alcC)
2240	*−2.09	*−2.27	−1.68	contig1999Oct161435_1677	Alcohol dehydrogenase I (alcA)
2400	*−2.35	*−2.32	−1.73	contig1999Oct161435_1677	Alcohol dehydrogenase I (alcA)
2177	*−1.76	*−1.69	−1.43	PCR	Aldehyde dehydrogenase (aldA)
Regulatory proteins					
1737	*−1.22	*−1.64	*−1.55	PCR	Acetyl-CoA synthetase (facA)
2559	−1.00	−1.15	−1.02	PCR	Acetate utilization regulator (facB)
1093	−1.04	*−1.39	*−1.45	PCR	Acetate utilization regulator (facB)
1089	−1.14	*−1.47	−1.22	PCR	Carbon catabolite represser (creA)
2155	*1.33	*1.37	*1.51	contig1999Oct161435_1531	Transcription factor (hacA)
Stress response genes					
460	−1.19	*−1.66	*−1.53	contig1999Oct161435_3948	Heat shock protein (hspA)
666	*−1.33	*−1.65	*−1.49	contig1999Oct161435_796	Heat shock protein (hspA)
2523	−1.07	*−1.27	*−1.22	contig1999Oct161435_796	Heat shock protein (hspA)
House-keeping genes					
2449	−1.18	*−1.41	−1.16	PCR	Actin (actA)
2291	−1.02	−1.56	*−1.32	PCR	β-Tubulin (benA)
606	1.05	1.02	1.04	contig1999Oct161435_1592	γ-Actin (actG)
766	1.04	1.09	1.12	contig1999Oct161435_1592	γ-Actin (actG)
Other genes of interest					
54	*−1.56	*−1.52	*−1.47	contig1999Oct161435_591	Aconitase
3247	*−2.18	*−2.57	*−3.10	contig1999Oct161435_1536	Aconitase
3404	*−2.12	*−2.65	*−3.20	contig1999Oct161435_591	Aconitase
3910	1.10	*1.83	*3.09	contig1999Oct161435_3477	α-Glucosidase precursor (maltase—agIA)
4070	1.17	*2.86	*4.06	contig1999Oct161435_3477	α-Glucosidase precursor (maltase—agIA)
1198	*−1.87	*−2.37	*−2.20	PCR	Catalase (catB)
2285	*−1.41	*−1.63	*−1.95	PCR	Chitin synthase (chsB)
3917	−1.07	N/F	*1.92	PCR	Chitin synthase (chsB)
4191	*1.40	*1.24	−1.01	PCR	Chitin synthase (chsE)

Table 2 (*continued*)

Spot No.	1 h	2 h	4 h	EST/PCR product	Gene
2286	*−1.89	*−2.85	*−3.19	PCR	Chitinase (chiB)
1197	*−1.23	*−1.78	*−1.87	PCR	Dehydroquinase (qutE)
3103	*1.35	*1.42	1.12	PCR	NADP-specific glutamate dehydrogenase (gdhA)

* denotes values that are statistically significant.

Table 3
Table of fold changes in expression for sequences encoding products with homology to ribosomal proteins

Spot No.	Log intensity	Normalized log ratio	Fold change	*p* value	Annotation
3633	7.31	−0.53	−1.70	0.00	40S ribosomal protein s27 type (*Schizosaccharomyces pombe*)
2804	5.81	−0.53	−1.70	0.00	60S ribosomal protein 32 (2,3-dihydroxy acid hydrolase)
2231	7.77	0.38	1.46	0.00	Ribosomal protein l18a.e.c13 (*Saccharomyces cerevisiae*)
1051	5.93	−0.75	2.10	0.00	Ribosomal protein ys7 homolog (*Emericella nidulans*)
1334	6.66	0.27	1.30	0.00	Ribosomal protein s14 (*Neurospora crassa*)
1301	7.19	0.33	1.38	0.00	60S ribosomal protein l21 (*Schizosaccharomyces pombe*)
3669	5.27	0.27	1.30	0.00	60S ribosomal protein l27a
513	5.87	0.25	1.29	0.00	Probable 60S ribosomal protein l11
1588	6.95	0.24	1.27	0.00	np_001010.1|prps 15a| ribosomal protein s15a
2137	5.15	0.23	1.26	0.00	Mitochondrial ribosomal protein l23 (*Kluyveromyces lactis*)
4326	5.81	0.22	1.25	0.00	40S ribosomal protein s30
778	5.51	0.22	1.24	0.00	60S ribosomal protein l23a
353	5.24	0.20	1.22	0.01	Probable 60S ribosomal protein l11
1188	6.52	0.20	1.22	0.01	60S ribosomal protein l27a
1060	6.80	0.20	1.22	0.01	40S ribosomal protein rs16 homolog
2425	6.48	0.18	1.20	0.01	Ribosomal protein l18a.e.c13 (*Saccharomyces cerevisiae*)

For 2-carbon metabolism, hexose phosphates needed for biosynthetic reactions are produced by gluconeogenesis using oxaloacetate from the glyoxylate cycle (see Fig. 5), which is converted into phosphoenolpyruvate by the action of phosphoenolpyruvate carboxykinase (*acuF*). In our glucose up-shift experiment, this enzyme shows an average of 2.24-fold down-regulation. Fig. 5 highlights the *A. nidulans* enzymes in the metabolic pathways involved in carbon and energy metabolism that are represented on the array. This diagram illustrates the down-regulation of the alcohol dehydrogenase and aldehyde dehydrogenase genes upon the addition of the more favoured carbon source glucose to the culture medium. This is in contrast to the diagram of DeRisi et al. (1997), which illustrates the diauxic shift in metabolism from anaerobic fermentation of glucose to the aerobic metabolism of ethanol. Our diagram also depicts the expected down-regulation of enzymes involved in the glyoxylate cycle and gluconeogenesis pathways in contrast to the up-regulation of these enzymes seen in the experiment of DeRisi et al.

4.6. Statistical analysis versus fold changes

Following the interpretation of the changes in gene expression that ensue upon glucose up-shift, in terms of our current understanding of the physiology and metabolic biochemistry of *A. nidulans*, we would like to close with some general observations on the quantitative interpretation of microarray data. Many published microarray experiments report fold changes for genes differentially expressed in control and experimental conditions (DeRisi et al., 1997; Desikan et al., 2001; ter Linde et al., 1999; Pérez-Amador et al., 2001; Sudarsanam et al., 2000). This is the simplest and most intuitive approach, but the threshold of greater than 2- or 3-fold is chosen arbitrarily and may be inappropriate. For example, if a greater than 2-fold change is selected and the condition under study does not affect any genes to the point of a 2-fold change, no genes will be selected and the experiment will have zero sensitivity. Equally, if the condition results in many genes changing dramatically, too many genes will be selected to usefully inform the design of follow-up experiments. Thus, using a fold change method without a clear biological justification is merely a blind guess (Draghici, 2002). Moreover, even a 3-fold change may not be statistically significant if the data show high variance. Indeed, Wurmbach et al. (2002) state that "microarray studies and appropriate numbers of replicates could identify genes (in the mouse brain) showing less than 2-fold regulation and that most regulated genes identified fell within this range."

Replication of the experiment is important to eliminate the problem of 'noise' inherent in microarray analyses (Ting Lee et al., 2000). Without replication, it is not possible to distinguish between true differences in gene expression and differences due to experimental variability. Arbitrarily dismissing genes with less than 2- or 3-fold expression changes may completely miss

Table 4
Known *A. nidulans* genes represented on the array (294)

abaA	Abacus aconidial regulatory protein
abfB	α-L-Arabinofuranosidase
accA	Acetyl-CoA carboxylase
aciA	Unknown regulated by amdA
acoB	Sporulation
actA	Actin
actG	γ-Actin
acuD	Isocitrate lyase
acuE	Malate synthase (glyoxomal)
acuF	Phosphoenolpyruvate carboxykinase
acuH	Acyl carnitine carrier
acvA	Antibiotic biosynthesis
adh3	Alcohol dehydrogenase 3
aflR	Sterigmatocystin biosynthesis regulation
agaA	Arginase
agdB	α-Glucosidase B
aglA	α-Glucosidase maltase
alcA	Alcohol dehydrogenase I
alcB	Alcohol dehydrogenase II
alcR	Alcohol regulatory protein
aldA	Aldehyde dehydrogenase
alkR	Alkane regulator
ama1	Terminal inverted repeat
amcA	Mitochondrial transport protein
amdA	Acetamidase reg DNA-binding protein
amdX	Binding regulatory protein
amyA	α-Amylase
amyB	α-Amylase
amyR	Amylase cluster transcriptional regulator
ankA	Cell cycle kinase (wee1)
anpC	Kex2-like dibasic endoprotease
apsA	Coiled-coil protein
apsB	Aminopeptidase B
argB	Ornithine carbamoyltransferase
aroC	Chorismate mutase
aroG	phe-inhibited DAHP synthase
aroM	Aromatic amino acid biosynthesis
aspB	Septin b
aspC	Septin c
aspnd1r	Antigen 1
atrA	ATP-binding cassette multidrug transporter
atrC	ATP-binding cassette multidrug transport
atrE	ATP-binding cassette transporter
AurA	Aureobasidin-resistance protein
awh11	Chaperone/heat shock protein
benA	β-Tubulin
bimA	Mitosis protein
bimB	Cell division protein
bimC	Kinesin-like protein
bimE	Negative regulator of mitosis
binC	Bafilomycin induced protein
brlA	Regulatory bristle protein
brlA	Regulatory protein (bristle a protein)
bzuA	Benzoate-*para*-hydroxylase
catA	Catalase A
catB	Catalase B
catC	Catalase C
cbf5	Centromere/microtubule-binding protein
cbhA	1,4-β-D-Glucan cellobiohydrolase
chi	Chitinase
chiA	Chitinase
chiB	Chitinase b?
chsA	Chitin synthase A
chsB	Chitin synthase B

Table 4 (*continued*)

chsC	Chitin synthase C
chsE	Chitin synthase
ciaA	Complex I intermediate associated protein
cipA	Concanamycin A—response to
citA	Citrate synthase
cmkA	Calmodulin-dependent protein kinase
cmkB	Calcium/calmodulin-dependent kinase B
cmkC	Calcium/calmodulin-dependent kinase
cnaA	Calmodulin-dependent phosphatase
cnxF	Molybdenum cofactor biosynthetic protein
cnxH	Molybdopterin synthase large subunit
cobA	Cytochrome *b*
cpa	cpa-like gene?
cpyA	Carboxypeptidase
creA	Carbon catabolite repressor
crnA	Nitrate transporter (nitrate permease)
csmA	Chitin synthase?
culA	scf complex protein
cysB	Cysteine synthase gene
cysD	*o*-Acetyl-L-homoserine sulphhydrylase
cytC	Cytochrome *c*
dap	Dipeptidyl aminopeptidase?
dewA	Spore-wall fungal hydrophobin
eRF	Eukaryotic polypeptide releasing factor
facA	Acetyl-coenzyme a synthetase
facB	Acetate utilization regulator
facC	Carnitine acetyl transferase
fadA	Guanine nucleotide-binding protein β subunit-like protein
fahA	Fumarylacetoacetate hydrolase
fasA	Fatty acid synthase, α subunit
fasB	Fatty acid synthase, β subunit
fksA	Putative 1,3-β-D-glucan synthase catalytic subunit
flbA	Developmental regulator
flbC	Putative zinc-finger protein
flbD	DNA-binding protein
fluG	Glutamate synthatase family of proteins
fmdS	Formamidase
gabA	Gaba permease
ganB	g protein α subunit
gatA	γ-Amino-*n*-butyrate transaminase
gdhA	NADP-specific glutamate dehydrogenase
gfdA	Glycerol-3-phosphate dehydrogenase
glnA	Glutamine synthetase
gltA	Glutamate synthase
gpdA	Glyceraldehyde-3-phosphate DH
gsdA	Glucose-6-phosphate DH
gstA	Glutathione *S*-transferase
hacA	Transcription factor
hapB	hapb
hapC	Transcription factor
hapE	DNA-binding factor
hemA	Mitochondrial precursor hemebiosynthesis
hex1	Perioxosomal gene?
hhfB	Histone H4.2
hhtA	Histone
hisHF	Glutamine amidotransferase:cyclase
hmgA	Homogentisate 1,2-dioxygenase
hogA	Map kinase
hpa2	hpaii-6, DNA fragment a
hscA	Chaperone protein hsp 70
hsp	Heatshock protein (hsp)
htaA	Histone H2A
htbA	Histone H2B
hxA	Hypoxanthine, xanthine dehydrogenase II

Table 4 (*continued*)

hxB	Hypoxanthine, xanthine dehydrogenase
hxB	Hypoxanthine, xanthine dehydrogenase I
hymA	Conidiophore development protein
icdA	Isocitrate dehydrogenase
Inu1	exo-Inulinase
IPNS	Isopenicillin n synthetase
kapR	cAMP-dependent protein kinase reg sub
karA	Karyopherin α
lamB	Lactam utilization protein
lipA	Sterigmatocystin biosynthesis lipase?
lsdA	Levansucrase precursor?
lysF	Homoaconitase, mitochondrial precursor
manA	Mannose-6-phosphate isomerase
mcsA	Methylcitrate synthase
mdr	Multidrug resistance protein
mdrA	Drug resistance protein
mdrA	Multi-drug resitant protein
med A	Medusa developmental regulator
metE	Homoserine *O*-acetyltransferase
metH	Cobalamin-independent methionine synthase
mit	Cytochrome oxidase subunit 1
mlpA	Myosin-related protein homolog
mns1A	er α-1,2-mannosidase?
mns1B	α-Mannosidase 1B
mpkA	Protein-tyrosine kinase?
mpkB	Mitogen-activated protein kinase
msd2	α-Mannosidase
msd2	α-Manosidase
mutA	Mutanase
nadA	Adenine deaminase
ndhE	NADH-ubiquinone oxidoreductase chain 5
niaD	Nitrate reductase
niiA	Nitrite reductase
nimA	g2-specific protein kinase
nimE	g2/mitotic-specific cyclin b
nimO	g1/s regulator
nimP	DNA polymerase epsilon homolog
nimT	m-phase inducer phosphatase
nimX	DNA replication licensing factor
nirA	Nitrogen assimilation transcription factor
nmrA	Nitrogen metabolite repression regulator
nsd	DNA-binding protein?
ntf2	Nuclear transport factor 2
nudE	Nuclear migration protein
nudF	Nuclear migration
nudG	Dynein light chain, cytoplasmic
oliC	Oligomycin resistance, ATP synthase protein 9
orlA	Osmotic-remediable lysis, *N*-acetylglucosamine req
otaA	osmotic-remediable lysis, *N*-acetylglucosamine req
oxiB	Cytochrome *c* oxidase polypeptide ii
oxpA	Adenylosuccinate synthetase
p5cr	Pyrroline-5-carboxylate reductase
pacA	Acid phosphatase
palA	pH signal transduction pathway gene
palB	Cysteine protease
palF	Ambiental pH regulatory system protein
palH	pH signal transduction
palI	Membrane components of signal transduction pathways for ambient ph regulation
panK	Pantothenate kinase
pdcA	Pyruvate decarboxylase
pelA	Pectate lyase fgsc4
penDE	Isopenicillin n acyltransferase
pepP	Prolidase
pgkA	Phosphoglycerate kinase
pgmB	Phosphoglucomutase

Table 4 (*continued*)

phacA	Cytochrome p450 monooxygenase
phoA	Acid phosphatase precursor
phyB	Phytase b
pinA	Peptidyl–prolyl *cis*/*trans* isomerase mitotic
pkaC	cAMP-dependent protein kinase
pkaR	cAMP-dependent protein kinase regulatory chain
pkiA	Pyruvate kinase
pksST	Putative sterigmatocystin biosynthesis polyketide synthase
pmaA	Plasma membrane H(+)ATPase
pmtA	Dolichyl-phosphate-mannose:proteinmannosyltransfer
pod40	ctd phosphatase-like protein
pphA	Protein phosphatase 2a
prnB	Proline permease
prtA	Alkaline protease
prtB	Putative aspartic protease
pyrE	Dihydroorotate dehydrogenase
pyrG	Uridine 5′-monophosphate synthase
pyroA	Pyridoxine synthesis
pyroB	Pyridoxine
qutA	Quinate pathway regulator
qutB	Quinate 5-dehydrogenase
qutC	Dehydroshikimate dehydratase?
qutE	Dehydroquinase
qutG	Quinate metabolism
qutR	Quinate repressor protein
Ras	ras-like protein
rbpE	RNA polymerase subunit
rcaA	Campothecin resistance protein
recA	recA
rhoA	GTPase
rhoC	GTPase
rnrA	Ribonucleotide reductase
rodA	Rodlet protein precursor
Rpl16a	Ribosomal protein
Rpl3	60S ribosomal protein
Rps16	Ribosomal protein
sagA	Aensitivity to DNA-damaging agents
salA	Aalicylate 1-monooxygenase
sAT	Aulfate reductase—3′-phosphoadenylyl
SC	Sulphate adenylyltransferase
schA	cAMP-dependent protein kinase-like
sconC	Sulphur metabolite repression control protein
sdeA	Stearic acid desaturase
sepA	Cytokinesis protein sepa
sepB	Chromosome segregation
sepH	Septation
sfaD	g-protein β subunit
sgdB	ras signaling?
sldA	Spindle assembly checkpoint protein
snaD	Suppressor gene of nuda1 mutation
sodA	Superoxide dismutase
sodVIC	Coatomer α subunit
sonA	Nuclear localization of cyclin b?
spoC1	Condium-specific protein
Stc	Sterigmatocystin biosynthesis
stcA	Sterigmatocystin biosynthesis polyketide
stcB	Serigmatocystin biosynthesis p450 monooxygenase
stcE	Sterigmatocystin biosynthesis putative ketoreductase
stcI	Sterigmatocystin biosynthesis lipase/esterase
stcS	Sterigmatocystin biosynthesis p450 monooxygenase
stcT	Sterigmatocystin biosynthetic gene cluster
stcV	Sterigmatocystin biosynthesis dehydrogenase
stcW	Sterigmatocystin biosynthesis monooxygenase
ste12	Homeodomain DNA-binding transcription factor
stuA	Cell pattern formation-associated protein

Table 4 (*continued*)

suaprga1	Regulation of penicillin biosynthesis
sudA	Chromosome scaffold protein
sudD	Extragenic suppressor genes of the bimd6 mutation
swoF	Myristoyl transferase
swoH	Nucleoside diphosphate kinase
tamA	Nitrogen regulatory protein
tbp	tata-box-binding protein
tbp	Transcription initiation factor tfiid (tata-box factor)
tcsA	Histidine kinase
thiO	Thioredoxin
tim	Zinc-finger-like protein
topA	Topoisomerase i
tpsA	Trehalose-6-phosphate synthase
treA	Acid trehalase precursor
treB	Trehalase
trpC	Anthranilate synthase
tubC	Tubulin η-2 chain
uapA	Uric acid-xanthine permease
uapC	Purine permease
uaY	Positive regulator of purine utilization
ubiA	Ubiquitin ubi1
usgS	Transmembrane protein
uvsB	Checkpoint protein
uvsC	Homologue of yeast rad51
uvsD	rad26 homolog putative
uvsF	Homology to DNA replication factor c
uvsH	Post-replication repair protein uvsh/nuva
uvsJ	DNA repair gene
vea1	Sexual development
verA	Versicolorin reductase
vpsA	Vacuolar biogenesis
vpsB	sec1-like protein
wetA	Spore-specific regulatory protein
xlnB	Xylanase
xlnC	Xylanase
xprF	Hexokinase-like protein

biologically important genes that have small fold changes. However, these relatively small changes in expression level may be highly significant statistically if they have been measured with high precision as a result of replication (Spruill et al., 2002). Puthoff et al. (2003) described a statistical method that relies on replicating experiments and takes into account the variability within and between treatment groups to distinguish gene expression changes caused by the experimental treatment from those attributable to biological and measurement variability. As a result, significant gene expression changes can be identified even if they are less than 2-fold in some or all replicates. They also confirmed their results using quantitative real-time PCR and compared their statistical analysis with a simple 2-fold cut off and obtained quite different results. Brown et al. (2001) compared microarray data published by DeRisi et al. (1997) with their own Northern data obtained for the same genes under similar growth conditions. They concluded that Northern analysis is "marginally more sensitive than microarray analysis at detecting low-level transcripts" but this may be due to unknown differences in the physiological conditions examined and/or the yeast strain used. The imposition of a 2-fold or 3-fold threshold to select genes for further study is an inappropriate legacy from early microarray studies, when possible sources of error in microarray experiments were not as well understood as they are now. Identification of intensity-dependent systematic errors has revealed that a considerable portion of a fold change may be due to systematic error, in particular at low intensities or for low expressed genes (Yang et al., 2002).

The statistical method used here combines normalization of 20 replicate hybridizations (leading to 40 replicate measurements for each probe). The normalization procedure involves corrections for location on the slide, thus eliminating artefacts that result from the robotic printing process, hybridization artefacts (including intensity-dependent systematic error), and other hybridization-specific systematic artefacts that are introduced during the production and use of microarrays. Thus, although the fold changes recorded are often relatively low, they have a high degree of statistical assurance, and have produced data that are congruent with our current view of carbon catabolite repression in the fungi.

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