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Phase-specific gene expression underlying morphological adaptations of the dimorphic human pathogenic fungus, *Coccidioides posadasii*

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Abstract

Coccidioides posadasii is a dimorphic fungal pathogen that grows as a filamentous saprobe in the soil and as endosporulating spherules within the host. To identify genes specific to the pathogenic phase of *Co. posadasii*, we carried out a large-scale study of gene expression in two isolates of the species. From the sequenced *Co. posadasii* genome, we chose 1000 open reading frames to construct a 70-mer microarray. RNA was recovered from both isolates at three life-cycle phases: hyphae, presegmented spherules, and spherules releasing endospores. Comparative hybridizations were conducted in a circuit design, permitting comparison between both isolates at all three life-cycle phases, and among all life-cycle phases for each isolate. By using this approach, we identified 92 genes that were differentially expressed between pathogenic and saprobic phases in both fungal isolates, and 43 genes with consistent differential expression between the two parasitic developmental phases. Genes with elevated expression in the pathogenic phases of both isolates included a number of genes that were involved in the response to environmental stress as well as in the metabolism of lipids. The latter observation is in agreement with previous studies demonstrating that spherules contain a higher proportion of lipids than saprobic phase tissue. Intriguingly, we discovered statistically significant and divergent levels of gene expression between the two isolates profiled for 64 genes. The results suggest that incorporating more than one isolate in the experimental design offers a means of categorizing the large collection of candidate genes that transcriptional profiling typically identifies into those that are strain-specific and those that characterize the entire species.

Keywords: Development; Spherules; Endospores; Microarray; Fungal pathogen; Population

1. Introduction

The dimorphic pathogenic fungus *Coccidioides posadasii* is found in arid regions of the Southwestern United States, Mexico, and Central and South America. It lives in the environment as a hyphal saprobe, reproducing by mitotic conidia. Alternatively, it can survive and reproduce in a mammal, as a parasitic, enlarging spherical cell, reproducing by mitotic endospores. This parasitic, spherule morphology is unique to *Coccidioides* and therefore appears to have evolved within the genus. Infection is caused when mammals inhale the air-dispersed asexual spores (arthroconidia), produced by the saprobic phase of the fungus. Within the lungs of the host, the barrel-shaped arthroconidia ($\sim 2 \times 4\,\mu m$) develop into multinucleate round cells (spherules) and grow isotropically to produce large parasitic cells (60–100 μm in diameter). The spherules undergo an elaborate process of endogenous wall growth and cytoplasmic compartmentalization. This developmental cascade

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culminates in the production and subsequent release of a multitude of endospores into the surrounding host tissue. Released endospores differentiate into new generations of endosporulating spherules, which can disseminate from the lungs to multiple other body organs (Galgiani, 1999). Upon the death and decay of the host, the fungus reverts to its saprobic, infectious, morphology (Maddy and Crecelius, 1967; Saubolle, 1996). This pattern of reversion to saprobic lifestyle appears to be the natural progression of the infection; direct transmission of the fungus between hosts has never been reported (Pappagianis, 1988).

The ability of arthroconidia of *Coccidioides* to grow and develop into spherules within the lungs of the host is a remarkable adaptation to a facultative pathogenic lifestyle. Despite the fundamental role the different morphological phases play in the course of infection, little is known about the novel gene regulation required for the transition from saprobic hypha to parasitic spherule, or for the transition from enlarging spherule to endospore production. The transformation between the saprobic and parasitic phases of *Coccidioides* can be recapitulated in culture by shifting the temperature from 30 °C to 39 °C in the presence of 20% CO_2 (Breslau and Kubota, 1964). The ability to induce both growth forms under laboratory conditions makes it possible to identify phase-specific genes. By using suppression subtractive hybridization (Diatchenko et al., 1999), Delgado et al. (2004) identified four genes whose expression is essentially restricted to the parasitic cycle. The use of DNA microarray technology to profile gene expression has enabled further identification of candidate genes that may be vital to the development of particular phases of the life cycle of pathogenic fungi, and enabled the analysis of global patterns of gene expression between developmental phases (e.g., Hwang et al., 2003). Although such studies explore the impact of developmental state upon gene expression, their generality has been limited by examining only a single genotype. We know that there is substantial intraspecific variation in expression profiles among isolates of the same fungal species. For example, significant differences in gene expression have been found among strains of *Paxillus involutus* (Le Quere et al., 2004) and *Saccharomyces cerevisiae* (Brem et al., 2002; Fay et al., 2004; Townsend et al., 2003), as well as differences in gene content between non-pathogenic and pathogenic *S. cerevisiae* strains (Winzeler et al., 2003). This is the first study of genome wide transcription in pathogenic fungi to examine differences between isolates.

In this study, we performed a large-scale analysis of gene expression in two isolates of *Co. posadasii* from one population, at three phases of their life cycle. We used the recently available genome sequence to design microarrays with spotted 70-mers representing 1000 manually annotated open reading frames (ORFs). By using this array we identified 92 genes that were significantly differentially expressed between pathogenic and saprobic phases in both fungal isolates. Furthermore, we found 43 genes with significant differential expression between the two investigated parasitic developmental phases. Intriguingly, differential gene expression between the two isolates was found for 64 genes. Our results challenge the inferences made by studies that examine a single pathogen genotype. Potentially, incorporating numerous isolates in the experimental design offers a means of categorizing the large collection of differentially expressed genes into transcriptional differences that are strain-specific and those that are species-wide. Many of the differences in gene expression observed in single-genotype studies may correspond to phenotypic variation in gene expression that is selectively neutral to, or incidental to, the functional questions of a given study. The research reported here sets the stage for further discovery of genes important to the evolution of the pathogenic morphology by examination of a larger collection of *Co. posadasii* isolates, and by investigation of the novel functions of the identified genes.

2. Materials and methods

2.1. Fungal material and culture conditions

The two *Co. posadasii* isolates used in this study are C735 and RMSCC Silveira. C735, the same isolate as used for the *Co. posadasii* genome sequencing project, originates from the Arizona population (M. Fisher, unpublished data). Silveira, the holotype of *Co. posadasii* (Fisher et al., 2002a), also originates from the Arizona population and is a widely used laboratory isolate. It was originally isolated in 1951 in the San Joaquin Valley, California, and must have been transported from Arizona to California in the host (Fisher et al., 2002b). In the experimental design, C735 is referred to as genotype 1 (G1) and Silveira as genotype 2 (G2). For each isolate, cultures of the saprobic and parasitic phase of the fungus were grown as previously described (Delgado et al., 2003) and fungal material was harvested at three developmental phases (Fig. 1). Saprobic phase cultures (H) were grown for 4 days at 30 °C in Converse medium (Converse, 1957). The cultures were harvested by suction filtration, washed once with sterile PBS after which RNA was immediately isolated (see below). Parasitic phase cultures were grown in Converse medium at 39 °C under 20% CO_2 and harvested based on morphology rather than at a specific time. Presegmented (S) and endosporulating spherules (S/E) were harvested by centrifugation for 5 min at 3000*g*, after which RNA was immediately isolated.

2.2. RNA extraction and amplification

Total RNA was extracted from single cultures of all three developmental phases (H, S, and S/E, Fig. 1) of each of the two isolates, using the modified RNeasy protocol with on-column DNase digestion (Qiagen, Chatsworth, CA) and stored at −70 °C until required. As a biological replicate, an independent growth and extraction was performed on the hyphal phase of the Silveira isolate (G2).

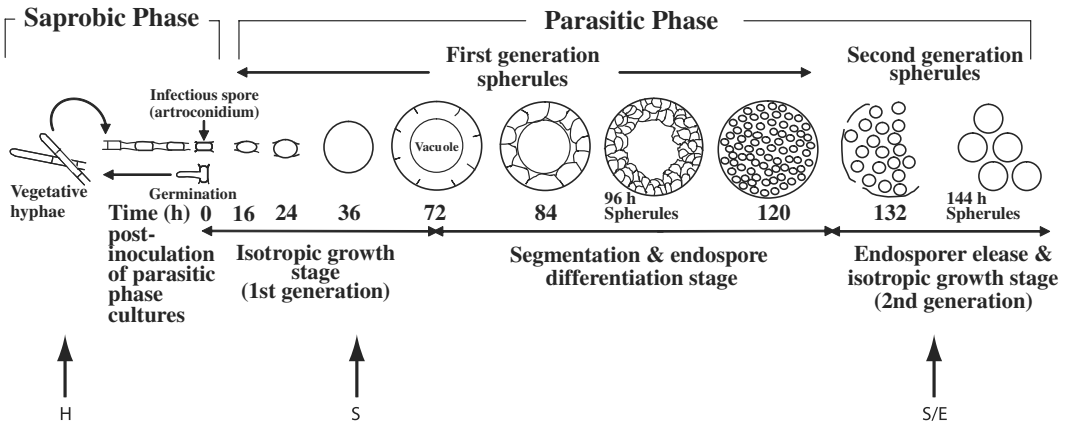

Fig. 1. Schematic presentation of the saprobic and parasitic phases of *Co. posadasii*. RNA was extracted from three developmental phases, H, hyphae, S, presegmented spherules, and S/E, spherules releasing endospores (after Delgado et al., 2003).

The purified RNA was checked for integrity and quality by agarose gel electrophoresis. Between 1 and 3 μg of total RNA were used for single rounds of amplification of antisense RNA (aRNA) by in vitro transcription with T7 RNA polymerase (Van Gelder et al., 1990) using the Amino Allyl MessageAmp™ aRNA kit (Ambion, Austin, TX) according to the manufacturer's instruction.

2.3. Annotation of *Co. posadasii* genome sequence

Annotation of *Co. posadasii* genome sequence relied on predictions of gene location and on homology with genes coding for known proteins. Genomic DNA sequences obtained from TIGR were blasted (BlastX) against the NR database from NCBI. *Co. posadasii* genes were also annotated by using a bidirectional BLAST search (tblastn) between total gene sets of *Co. posadasii* and *Neurospora crassa*, the best annotated filamentous ascomycete (Galagan et al., 2003; Mannhaupt et al., 2003): genes were judged to be orthologous to *N. crassa* genes only when the blast of both of the genes in a pair resulted in the best hit to each other, and *E* values were less than 10^{-5}.

2.4. Construction of *Co. posadasii* microarrays

To construct a gene-specific microarray of the *Co. posadasii* genome, we designed 70-mer oligonucleotide array elements. The ArrayOligoSelector software package (Bozdech et al., 2003) was used to design a DNA microarray composed of 6031 manually annotated ORFs. The program optimizes the oligo selection for each open reading frame based upon uniqueness in the genome, sequence complexity, lack of self-binding, GC content, and proximity to the 3′ end of the gene. For the composition of the microarray, 1000 ORFs were selected (by authors R.S. and B.G.) to include all reliably annotated genes with homology-predicted function related to antigenicity, morphology, differentiation, development, nitrogen metabolism, and cell wall synthesis. In addition, housekeeping genes with predicted or previously verified constitutive expression were included as controls. For these 1000 ORFs, representative 70-mers were synthesized (Illumina, San Diego, CA), resuspended in 3× SSC to a final concentration of 20 μM, and spotted in duplicates onto UltraGAPS™ coated slides (Corning, NY). Eight of 20 μM ArrayControl Sense Oligo spots, complementary to eight ArrayControl RNA spikes (Ambion, Austin, TX, USA), were included in duplicate. Previous studies have shown that the intraspecific variability in coding regions of *Co. posadasii* is very low, with the proportion of variable sites of nuclear genes being 1% or less (Johannesson et al., 2004, 2005; Koufopanou et al., 1997). Therefore we consider this 70-mer array, designed from the C735 genome sequence, to be appropriate for the analyses of expression of multipe *Co. posadasii* isolates, especially when originating from the same population.

2.5. Hybridization and image acquisition

The experimental design to detect gene regulation differences between isolates and developmental phases generally followed the guidelines of Townsend and Taylor (2005), and is depicted in Fig. 2. Hybridizations were performed in dye swap pairs, graphed as double-headed arrows in Fig. 2, in which 5–10 μg each of amino-allyl aRNA samples from two conditions were coupled to either of the post-labelling reactive dyes Cy3 or Cy5 (Amersham Biosciences, Uppsala, Sweden). Independent cDNA syntheses and amplifications of total RNA samples were performed for each dye swap replicate pair. The coupling was performed by using the reagents provided by the Amino Allyl MessageAmp™ aRNA kit (Ambion, Austin, TX) according to the manufacturer's instructions. All hybridizations were carried out using the Universal Microarray Hybridization kit (Corning, NY). An Axon GenePix 4000B scanner (Axon Instruments, CA, USA) was used to acquire images of microarrays, and GenePix Pro 4.1 software was used to quantify hybridization signals.

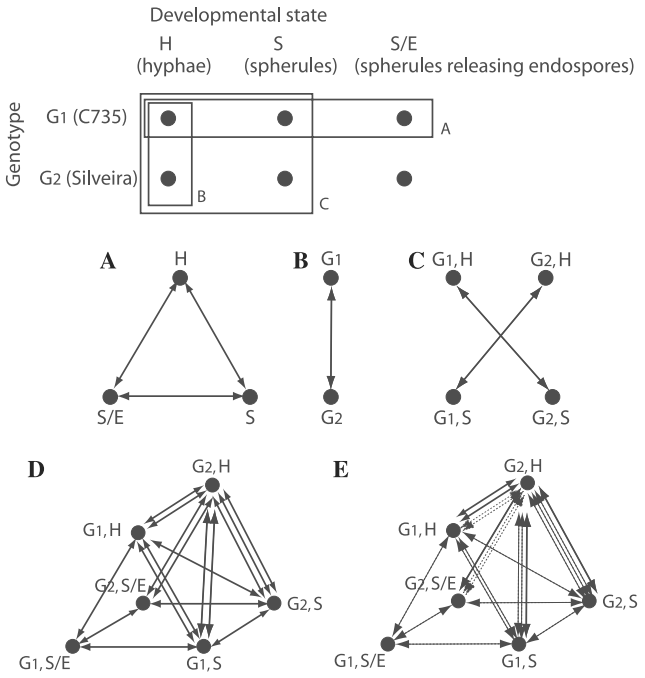

Fig. 2. Experimental design of this study. Circular nodes represent samples of total RNA that were harvested from three developmental phases of the two genotypes. Each double-headed arrow represents a dye swap replicate pair, i.e., two competitive hybridizations between aRNA amplified from the total RNA samples and labelled with the alternative dyes Cy3 and Cy5. (A) Component of design for comparison of the three developmental phases for each genotype. (B) Component of design for the comparison of the two genotypes for each developmental phase, and (C) Component of design for the comparison of hyphae and presegmented spherules for the two genotypes. (D) Multigraph of comparisons performed in this study using the six total RNA samples, not including the biological replicate. (E) Multigraph in which solid lines indicate additional comparisons using the biological replicate of sample G2, H.

2.6. Data analysis

A gene was chosen for further analysis if the mean target intensity for the probe spot, in at least one of the two hybridizations in the dye swap replicate pair, was three standard deviations greater than the mean background intensity, and if pixel saturation in the spot was less than 2%. Most duplicated spots had very similar intensities and ratios, and for each dye swap replicate pair we retained the ratio of hybridization intensities only from the morphologically sound spot with the highest overall fluorescence intensity. Following linear global normalization, Bayesian analysis of gene expression levels (BAGEL) was used to infer a relative gene expression level and variance for each gene at each experimental condition (Townsend and Hartl, 2002).

2.7. Logistic regression of the empirical power to detect differences in gene expression level

To provide a descriptor of the power of the experimental design, logistic regressions of the probability of a difference in expression being statistically significant as a function of estimated expression level (Townsend and Taylor, 2005) were performed on select comparisons with one, two, and three direct dye swap replicate pairs. Simulations have demonstrated that such logistic regressions, plotting the Bayesian estimated ratios on the "independent" x-axis, approximate regressions where the actual gene expression ratio was plotted on the x-axis (Townsend, 2004). As examples of comparisons comprising one, two, and three dye swap replicate pairs we used the node comparisons (G1, S) vs. (G1, S/E), (G1, H) vs. (G2, H), and (G2, H) vs. (G2, S), respectively (Fig. 2D). The logistic model was $\log_e p/(1-p) = mx + b$, where p is the probability of an affirmative significance call, x is the $\log_2$ factor of difference in gene expression, m is the slope of the logistic curve, and b is the intercept.

2.8. Quality control of microarray

As a quality control of the method of amplifying and hybridizing the RNA, hierarchical clustering of the normalized data was performed to verify that the dye swap replicate comparisons of the same nodes cluster together (Sandrine Dudoit, Division of Biostatistics, UC Berkeley, personal communication). Furthermore, the result from the microarray study was compared with published information on relative expression levels of genes from saprobic and parasitic cultures of isolate C735 (Delgado et al., 2004; Guevara-Olvera et al., 2000; Hung et al., 2002).

2.9. Identification of putative antigens

Putative antigens were identified based on the following criteria: (1) induced expression during parasitic growth in both isolates, (2) predicted localization (extracellular or GPI anchored), and (3) major histocompatibility complex II (MHC II) binding predictions. Localization of proteins was determined using the SignalP server at http://www.cbs.dtu.d+k/services/SignalP/#submission based on the algorithms of Bendtsen et al. (2004). Putative MHC Class-II binding regions in a potential antigenic sequence were determined using the quantitative matrices derived from the published literature by Sturniolo et al. (1999), and Singh and Raghava (2001), (http://bioinformatics.uams.edu/mirror/propred/).

3. Results

3.1. The power of the design

Logistic regression of the affirmative significance call against estimated fold change provides a method to characterize the acuity with which the experiment identified small but important changes in gene expression. In the circuit design (Fig. 2), comparisons between some pairs of samples had greater numbers of direct replicate hybridizations than other pairs of samples. Genes with low fold-changes between pairs of samples are expected to be more likely to be identified as significantly differentially expressed when

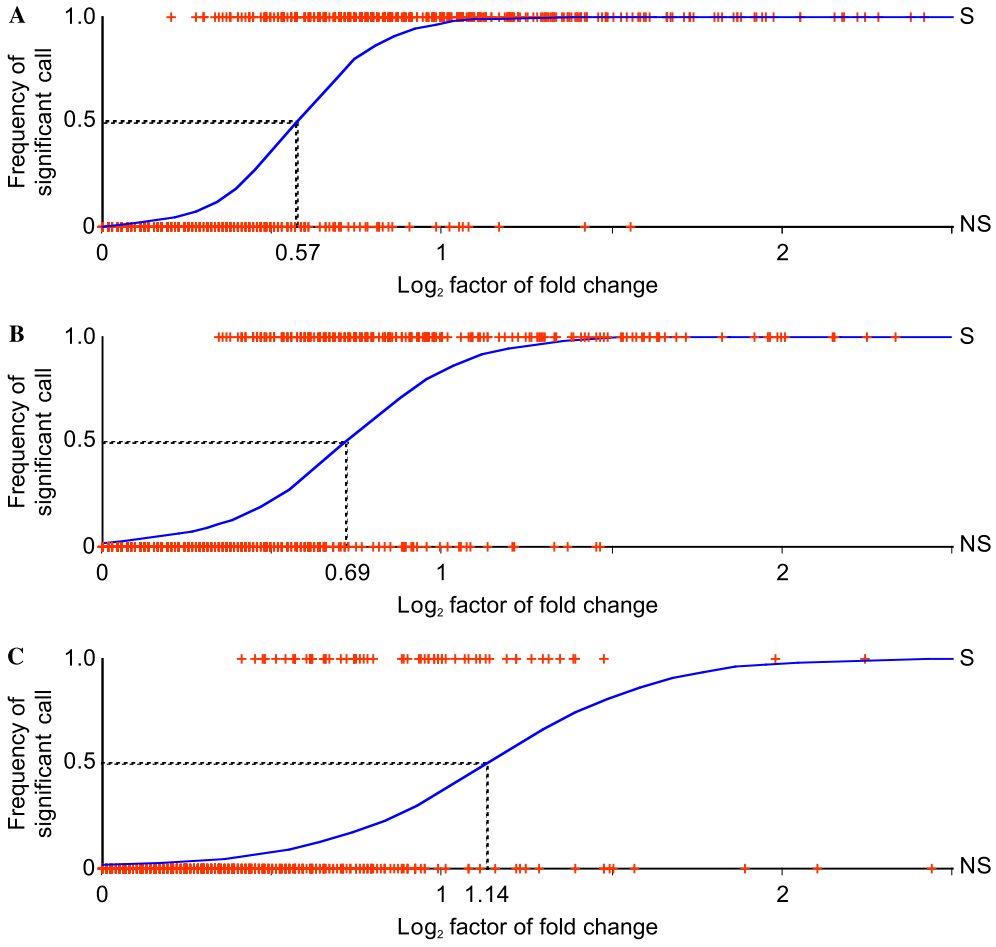

Fig. 3. Logistic regressions of the probability of a significant call as a function of estimated expression level. Plus symbols (+) are plotted at estimated expression levels from simulated data using a Bayesian analysis of gene expression level with additive small error terms. Each plus symbol is placed on the abscissa at the estimated expression level, either at the top of the plot (significant, S), or at the bottom (not significant, NS). (A) Comparison (G2, H) vs. (G2, S), with three direct dye swap replicate pairs, for which the factor of gene expression at which 50% of estimated differences were identified as significant (GEL_{50}) was 1.5-fold. The model has a highly significant fit ($\chi^2 = 146.1$, $p < 0.0001$). The estimated intercept for the log odds, b, of a significant call versus no significant call is -4.6 ($p < 0.0001$), and the estimated slope with $\log_2$ factor of difference in gene expression, m, is 7.9 ($p < 0.0001$). (B) Comparison (G1, H) vs. (G2, H), with two direct dye swap replicate pairs, for which $GEL_{50} = 1.6$-fold. The model has a highly significant fit ($\chi^2 = 644.3$, $p < 0.0001$). The estimated intercept for the log odds, b, of a significant call versus no significant call is -4.6 ($p < .0001$), and the estimated slope with $\log_2$ factor of difference in gene expression, m, is 7.9 ($p < 0.0001$). (C) Comparison (G1, S) vs. (G1, S/E), with one direct dye swap replicate pair, for which $GEL_{50} = 2.2$-fold. The model has a highly significant fit ($\chi^2 = 423.3$, $p < 0.0001$). The estimated intercept for the log odds, b, of a significant call versus no significant call is -4.3 ($p < 0.0001$), and the estimated slope with $\log_2$ factor of difference in gene expression, m, is 6.1 ($p < 0.0001$).

those pairs have been compared with a greater numbers of direct replicates (Townsend and Taylor, 2005). Befittingly, the factors of gene expression at which 50% of estimated differences were identified as significant were 2.2-, 1.6-, and 1.5-fold for one, two, and three direct dye swap replicate pairs, respectively (Fig. 3). Thus, where greater direct replication was applied, greater resolution of small differences in gene expression resulted.

3.2. Genes differentially expressed between developmental phases

The data revealed several categories of genes showing induced expression in the saprobic phase compared with the parasitic phases. A significantly higher expression in mycelia than in presegmented spherules was found for 263 genes, of which 92 had a significantly higher expression in mycelia in both isolates ($p < 0.05$, Fig. 4A). Of these 92, 62 also showed significantly higher expression in mycelia compared to endosporulating spherules in C735, and 42 showed this pattern in Silveira. Taken together, 27 genes were significantly upregulated in mycelia compared to both presegmented and endosporulating spherules in both isolates ($p < 0.05$). These genes, and the magnitude of expression difference, are found in Table 1A. A relatively large proportion of the genes with saprobic phase-induced expression shows no homology to any known protein, but the group also includes an organelle-specific gene (e.g., cole_406, Woronin body major protein), housekeeping genes (e.g., 8x_1367_9g, Acetylstransferase), and members of several other functional categories (Fig. 5).

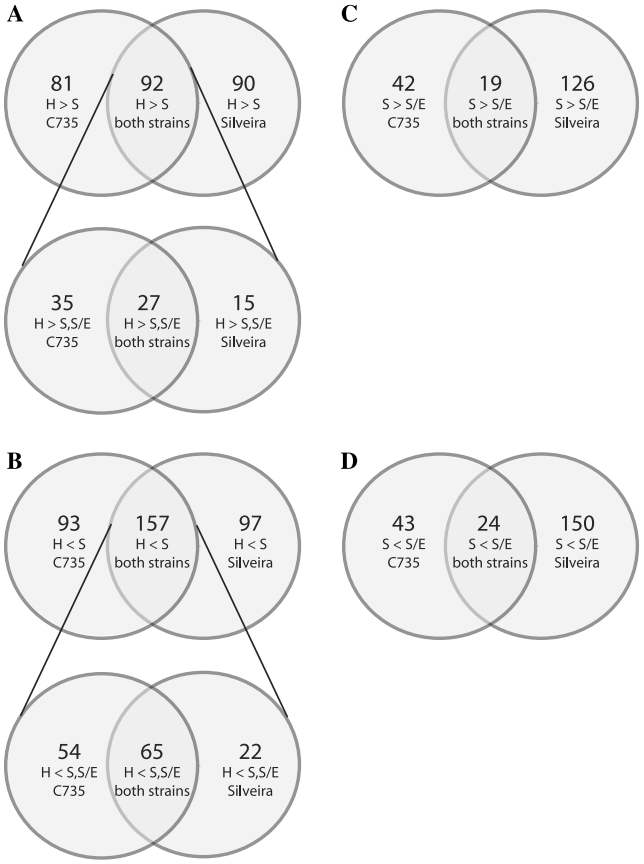

Fig. 4. Venn diagram showing the number of genes within the unique and shared sets of phase-specific genes for the two isolates ($p < 0.05$). (A) Genes upregulated in the saprobic phase (H) as compared to parasitic (S, S/E). (B) Genes upregulated in parasitic phases (S, S/E) as compared to saprobic phase (H). (C) Genes upregulated in presegmented spherules (S) as compared to endosporulating spherules (S/E). (D) Genes upregulated in endosporulating spherules (S/E) as compared to presegmented spherules (S).

A higher expression in presegmented spherules than in mycelia was found for 347 genes, of which 157 had a significantly higher expression in spherules than in mycelia in both isolates ($p < 0.05$, Fig. 4B). Of the 157, 65 genes were significantly upregulated in both presegmented and endosporulating spherules as compared to mycelia in both isolates ($p < 0.05$; Table 1B). This group contains genes of diverse functional groups, ranging from genes categorized as antigens to housekeeping genes (Fig. 5). One gene that was found to be highly induced in the parasitic phases of the life cycle is cole_48, homologous to nitrate reductase (*nir*, Fig. 6A). This gene is likely to be an important virulence factor in *Coccidioides*, as previously has been suggested for *Mycobacterium* species (Weber et al., 2000). Another group of genes upregulated in the parasitic phase includes genes involved in the metabolism of lipids, e.g., cole_64, CDP-phosphatidyltransferase, cole_389, phosphoinositide specific phospholipase C (this gene is now listed as, 1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase 1 in Table 1B. One of the names should be changed), cole_9, triglyceride lipase, and an unknown protein believed to be involved in ergosterol biosynthesis, cole_85 (Figs. 6 B–E), extending previous observations that spherules contain a higher proportion of lipids than saprobic phase tissue (Wheat et al., 1977). Furthermore, several genes potentially involved in the response to environmental stresses (e.g., two enzymes involved in the synthesis of the disaccharide trehalose: cole_119, trehalose synthase and 8x_1118_14g, trehalose-6-phosphate phosphatase, and cole_548, an osmotic sensitivity map kinase, Figs. 6 F–H) were found to be upregulated in the parasitic phase in both isolates.

When comparing the two parasitic phases, presegmented and endosporulating spherules, we found 19 genes with a significantly higher expression in presegmented as compared to endosporulating spherules in both isolates, and 24 genes with a significantly higher expression in endosporulating than in presegmented spherules in both isolates ($p < 0.05$; Figs. 4C and D; Table 1C and D). The gene that showed the greatest increase in expression in presegmented as compared to endosporulating spherules (Table 1C) codes for a protein involved in drug resistance, while the three genes showing the largest increase in expression in endosporulating compared to presegmented spherules (Table 1D) all lack homology to any known gene.

Differential expression was not detected for 65 genes between any of the included samples. This group contained genes coding for both uncharacterized hypothetical proteins and housekeeping genes (Fig. 5).

3.3. Identification of putative antigens

Based on the criteria given above, 13 genes were identified as coding for putataive antigenic proteins (Table 2). In validation of this method, two genes coding for proteins already shown to be immunoreactive were identified. The secreted chitinase 1 has been shown to react with patient anti-*Coccidioides* complement-fixing (CF) antibody and it is a valuable aid in the serodiagnosis of coccidioidomycosis (Reichard et al., 2000). Furthermore, the expression library antigen has been found to protect BalB/C mice from a lethal intraperitoneal challenge of *Co. posadasii* (Ivey et al., 2003). In addition to these two genes, 11 putative antigens were identified, some of which are known to be immunoreactive in other species. For instance, cole_383 has homology to a known allergen from the fungal respiratory pathogen *Aspergillus fumigatus* (Crameri, 1998) and cole_381 is homologous to an IgE binding protein also from *A. fumigatus*. One gene, 8x_1304_22g, appears to be unique to *Coccidioides*. This predicted protein has no homology to any known protein in the NR protein database and may thus be helpful in the serodiagnosis of coccidioidomycosis. Future studies will focus on the production of recombinant protein and an evaluation of its protective efficacy in a murine model.

Table 1
Phase-regulated genes in *Co. posadasii*

Gene name	Annotation[a]	Accession no of ortholog	(*E* value)[b]	Fold change[c]
(A) Saprobic phase induced genes (phase 1 vs. phase 2 and 3)				
8x_1342_4g	Unknown extracellular protein	n.a.	n.a.	5.81 (S1/S2)
8x_1254_2g	GABA permease	EAL86230	4 e−23	5.16 (C1/C3)
cole_101	Secretory phospholipase A2	BAD01581	9e−46	4.39 (C1/C3)
8x_1250_3g	Hypothetical protein	EAA73565	5e−60	4.37 (C1/C2)
cole_36	Kinesin light chain	ZP_00674577	1e−45	3.92 (C1/C2)
8x_513_10g	Unknown transmembrane protein	n.a.	n.a.	3.89 (C1/C3)
8x_1097_29g	Extracellular matrix protein	AAR06609.1	2e−15	3.67 (C1/C3)
8x_1376_9g	Acetyltransferase	EAL90534	3e−65	3.62 (S1/S3)
8x_1132_23g	BYS1 domain protein	EAL86105	1e−43	3.59 (C1/C3)
8x_1228_1g	Unknown	n.a.	n.a.	3.4 (S1/S3)
cole_401	*O*-methyltransferase	EAL91897	2e−74	3.29 (C1/C2)
cole_415	Nitrogen assimilation transcription factor	XP_657702	3e−70	2.89 (C1/C2)
cole_403	Polyketide synthase	AAR90275	9e−86	2.83 (S1/S3)
8x_1091_21g	MFS multidrug efflux transporter	EAL94082	4e−109	2.79 (S1/S3)
cole_293	Beta-glucosidase	AAB67972	0.0	2.79 (C1/C2)
8x_1386_7g	CFEM domain protein	EAL89245	9e−15	2.78 (S1/S3)
8x_1154_12g	Ada regulatory protein	EAL91960	6e−37	2.71 (C1/C3)
8x_592_16g	Extracellular leucine aminopeptidase	AAS76670	1e−108	2.69 (C1/C2)
cole_277	Beta-*N*-acetylglucosaminidase	AAU29327	2e−141	2.56 (C1/C3)
8x_1106_6g	Unknown extracellular protein	n.a.	n.a.	2.41 (S1/S3)
8x_517_2g	Unknown GPI-anchored protein	n.a.	n.a.	2.37 (C1/C2)
cole_406	Woronin body major protein	XP_662299	3e−65	2.23 (C1/C3)
8x_1117_1g	Phosphatotransferase family protein	EAL91121	4e−23	2.15 (C1/C2)
cole_43	Copper amine oxidase	EAL86351	8e−130	2.08 (S1/S2)
8x_415_10g	Glycosyltransferase family protein	CAC28725	4e−98	1.87 (S1/S3)
8x_1301_14g	Transporter protein	EAL91822	2e−94	1.86 (S1/S2)
cole_532	BRT1	AAK73279	4e−66	1.81 (S1/S2)
(B) Pathogenic phase induced genes (phase 2 and 3 vs. phase 1)				
cole_48	Nitrate reductase	EAL91563	2e−124	6.32 (C3/C1)
cole_252	Protein transport protein SEC31	AAV59730	8e−111	6 (C3/C1)
cole_1	COP9 signalosome complex	EAL92898	6e−155	5.67 (C3/C1)
cole_553	Two-component histidine kinase Fos-1	AAK27436	0.0	4.65 (S2/S1)
cole_64	CDP-phosphatidyltransferase	EAL89620	0.0	4.61 (C3/C1)
cole_409	Peptide synthase	EAL90366	1e−116	4.48 (C3/C1)
cole_103	Carboxypeptidase kex1	EAL90223	1e−138	4.45 (C2/C1)
8x_1147_26g	Expression library immunization antigen	AAO62547	3e−63	4.27 (S3/S1)
cole_350	Developmental regulator FlbA	EAL93413	1e−162	4.14 (S2/S1)
cole_226	SH3 and dedicator of_cytokinesis 1	EAL88144	9e−126	4.07 (S3/S1)
cole_592	M-phase inducer phosphatase	EAL88671	1e−122	3.95 (C3/C1)
cole_232	Calpain-like protease PalBory	EAL88057	8e−147	3.92 (S2/S1)
cole_521	Histidine containing phosphotransmitter	EAL89760	3e−41	3.86 (S2/S1)
cole_65	Ccr4-Not transcription complex subunit	EAL92557	1e−147	3.84 (C3/C1)
cole_499	Serine/Threonine protein kinases	EAL91429	2e−63	3.7 (S3/S1)
cole_46	Calcium transporting ATPase	AAF90186	0.0	3.69 (C2/C1)
cole_51	Magnesium ion-transporting ATPase	AAN33249	3e−60	3.62 (S2/S1)
cole_442	Rho guanyl nucleotide exchange factor	EAL91853	6e−156	3.59 (C3/C1)
cole_341	Serine/threonine protein kinase	EAL85809	3e−92	3.46 (C2/C1)
cole_544	Protein kinase (Chm1)	EAL92004	8e−109	3.46 (C2/C1)
cole_60	Cytokinesis regulator (Byr4)	EAL85866	2e−73	3.37 (S3/S1)
cole_115	Adenylate cyclase	AAS01025	0.0	3.37 (C2/C1)
8x_1346_20g	Alpha-1. 2-mannosyltransferase	CAC18268	3e−143	3.34 (C2/C1)
8x_1118_14g	Trehalose-6-phosphate phosphatase	AAO72737	0.0	3.27 (S3/S1)
8x_363_16g	Hypothetical extracellular protein	XP_329041	2e−25	3.22 (C3/C1)
cole_385	Tgf beta receptor associated protein 1	CAF32140	2e−128	3.17 (C3/C1)
cole_49	Voltage-gated chloride channel (ClcA)	EAL93186	2e−86	3.16 (S2/S1)
cole_389	1-Phosphatidylinositol-4.5-bisphosphate phosphodiesterase 1	EAL90657	2e−104	3.13 (S3/S1)
8x_1141_28g	Unknown intracellular protein	n.a.	n.a.	3.12 (C2/C1)
cole_85	Oxysterol binding protein (Orp8)	EAL93011	2e−103	2.96 (C3/C1)
cole_15	Beta-*N*-acetylglucosaminidase	EAL85175	0.0	2.94 (S2/S1)
cole_6	AP-1 adaptor complex subunit gamma	EAL88315	0.0	2.92 (C2/C1)
cole_187	Associated with ferric reductase; Utr1 protein	EAL87233	1e−114	2.89 (C2/C1)
cole_235	Heat shock protein 78	AAO49455	0.0	2.87 (C3/C1)

(*continued on next page*)

Table 1 (*continued*)

Gene name	Annotation[a]	Accession no of ortholog	(*E* value)[b]	Fold change[c]
8x_1164_1g	Vacuolar endopolyphosphatase	EAL90476	2e−74	2.85 (S3/S1)
cole_1221	Zinc-dependent alcohol dehydrogenase	ZP_00625270	2e−98	2.83 (S2/S1)
cole_447	F-box and WD repeat-containing protein	EAL93500	9e−61	2.82 (C2/C1)
8x_1161_23g	Hypothetical extacellular protein	XP_662919	6e−93	2.81 (C2/C1)
cole_599	Membrane bound C2 domain protein	EAL84710	2e−165	2.68 (C2/C1)
cole_157	Exocyst complex component (sec3)	EAL88146	2e−55	2.66 (C3/C1)
cole_493	Glutaryl-CoA dehydrogenase	EAL92964	1e−76	2.65 (C2/C1)
cole_244	*Sac*I domain protein	EAL93963	1e−125	2.64 (C3/C1)
cole_119	Trehalose synthase (Ccg-9)	EAL92373	4e−60	2.62 (S2/S1)
cole_25	Phospholipid-translocating P-type ATPase	EAL85644	6e−154	2.58 (C2/C1)
cole_523	Aldehyde reductase	AAL27089	2e−180	2.56 (C2/C1)
8x_1196_14g	Chitinase 7	AAR18252	0.0	2.52 (S3/S1)
cole_362	Endoglucanase	EAL86677	5e−72	2.51 (S2/S1)
cole_374	4-Hydroxyphenylpyruvate dioxygenase	JC4215	0.0	2.51 (C2/C1)
cole_336	Bud neck involved protein BNI4	AAT73074	7e−10	2.5 (S3/S1)
cole_132	Matrix AAA protease MAP-1	EAL87365	3e−123	2.48 (S2/S1)
cole_597	Serine/threonine protein kinase	EAL87038	1e−107	2.45 (S2/S1)
cole_2	SNI2 protein	EAL92477	0.0	2.42 (C2/C1)
cole_596	SUMO ligase SizA	EAL85515	9e−93	2.4 (C3/C1)
cole_580	Dipeptidyl aminopeptidase (Ste13)	EAL92790	5e−104	2.38 (C3/C1)
cole_88	Ste16 protein	AAW46807	9e−115	2.36 (C3/C1)
cole_571	Patched sphingolipid transporter	EAL88849	8e−179	2.34 (S2/S1)
cole_268	Alpha-amylase AmyA	EAL87420	2e−40	2.34 (C3/C1)
cole_548	Osmotic sensitivity map kinase	CAD28436	6e−64	2.27 (S2/S1)
Chs1	Chitin synthase 1	AAF82801	0.0	2.21 (S2/S1)
cole_503	Mannosyltransferase	XP_658333	2e−66	2.13 (S2/S1)
cole_9	Triglyceride lipase-cholesterol esterase	EAL91703	8e−151	2.04 (C2/C1)
cole_236	GTP cyclohydrolase	EAL90662	4e−123	2.02 (C3/C1)
cole_63	Extragenic suppressor of the bimD6 mutation	EAL93704	0.0	1.96 (C3/C1)
cole_227	Acetate regulatory DNA binding protein FacB	EAL90683	4e−56	1.83 (S2/S1)
8x_1089_9g	Hypothetical protein	EAL87983	2e−47	1.44 (S2/S1)
(C) Genes upregulated in presegmented (phase 2) versus endospulating spherules (phase 3)				
8x_1254_12g	Fluconazole resistance protein	CAD70375	5e−155	3.41 (S2/S3)
cole_222	Sodium P-type ATPase	CAB65298	0.0	2.76 (C2/C3)
cole_269	Proteasome component (Ecm29)	EAL88508	5e−83	2.28 (S2/S3)
cole_383	Cell wall glucanase	EAL92657	3e−67	2.18 (C2/C3)
cole_265	YlTSR1; involved the translocation pathway of secretory proteins	XP_504984	4e−22	2.1 (C2/C3)
8x_1134_19g	Stress response protein rds1p	CAD21425	2e−22	2.07 (S2/S3)
cole_98	Aflatoxin efflux pump AFLT	AAS89998	2e−92	2 (C2/C3)
cole_80	Pirin; iron-binding nuclear protein	EAL85220	8e−140	1.92 (S2/S3)
8x_1141_29g	GABA permease	EAL84297	2e−46	1.89 (S2/S3)
cole_168	High-affinity nickel-transport protein family	EAL93179	6e−59	1.87 (S2/S3)
cole_553	Two-component histidine kinase Fos-1	AAK27436	0.0	1.87 (S2/S3)
cole_251	Sterol delta 5.6-desaturase; ERG3	EAL85525	3e−103	1.87 (C2/C3)
cole_21	Alkaline phosphatase	EAL92183	3e−130	1.69 (C2/C3)
cole_49	Voltage-gated chloride channel (ClcA)	EAL93186	2e−86	1.6 (S2/S3)
8x_513_10g	Unknown transmembrane protein	n.a.	n.a.	1.51 (C2/C3)
cole_454	Mitotic check point protein (Bub2)	EAL88006	9e−81	1.47 (C2/C3)
8x_1186_23g	Endosomal cargo receptor (Erv14)	EAL88584	2e−59	1.47 (C2/C3)
8x_1367_7g	Calcium permease family membrane transporter	EAL84615	0.0	1.46 (C2/C3)
cole_45	Guanine nucleotide binding protein	EAL87348	0.0	1.4 (C2/C3)
(D) Genes upregulated in endosporulating (phase 3) versus presegmented spherules (phase 2)				
8x_1336_4g	Unknown	n.a.	n.a.	7.2 (S3/S2)
8x_1223_6g	Unknown	n.a.	n.a.	4.88 (S3/S2)
8x_260_1g	Unknown extracellular protein	n.a.	n.a.	4.77 (S3/S2)
cole_330	Chitinase	AAA92643	0.0	3.01 (S3/S2)
8x_257_1g	Retrotransposon; Ylt1	XP_504795	3e−29	2.4 (C3/C2)
8x_364_16g	Wax synthase like protein	BAB08549	1e−09	2.28 (S3/S2)
8x_1236_30g	Phosphotidylinositol kinase	EAL91349	0.0	2.14 (S3/S2)
cole_76	Proteasome regulatory particle subunit Rpt1	EAL92793	6e−150	1.99 (S3/S2)
8x_1127_12g	Hypothetical protein; RTA1 domain protein	EAA76585	8e−67	1.99 (C3/C2)
8x_595_14g	Endo-1.3(4)-beta-glucanase	EAL91216	9e−61	1.94 (C3/C2)
cole_90	D-xylulose 5-phosphate/D-fructose 6-phosphate phosphoketolase	EAL92505	3e−117	1.93 (C3/C2)
8x_1312_12g	Phosphate-repressible phosphate permease	EAL84983	2e−129	1.92 (C3/C2)

Table 1 (*continued*)

Gene name	Annotation[a]	Accession no of ortholog	(*E* value)[b]	Fold change[c]
8x_1139_50g	tRNA (adenine-*N*(1)-)-methyltransferase	EAL89520	3e−102	1.75 (S3/S2)
8x_1386_15g	Beta-1.4-mannosyltransferase	AAX31662	2e−138	1.74 (C3/C2)
8x_476_15g	Putative secreted protein	BAC68793	9e−35	1.62 (C3/C2)
cole_58	Fimbrin	EAL93040	7e−149	1.54 (S3/S2)
cole_381	IgE binding protein	EAL84222	2e−36	1.54 (S3/S2)
8x_502_17g	Actin-related protein RO7	EAL92259	2e−132	1.53 (C3/C2)
8x_553_1g	Unknown	n.a.	n.a.	1.45 (S3/S2)
8x_1168_2g	Oxyreductase-like protein	ABA38732	1e−123	1.44 (S3/S2)
cole_538	Orotate phosphoribosyltransferase (OPRT)	O93849	4e−108	1.39 (S3/S2)
cole_216	Adenylylsulfate kinase	EAL90409	5e−84	1.39 (C3/C2)
cole_533	DNA/RNA non-specific nuclease	AAK62983	4e−164	1.34 (S3/S2)
8x_1304_22g	Amine oxidase	EAL86183	5e−50	1.33 (C3/C2)

[a] When possible, localization of proteins was determined using the SignalP server at http://www.cbs.dtu.d+k/services/SignalP/#submission based on the algorithms of Bendtsen et al., 2004.

[b] *E* value ($<1 \times 10^{-6}$) obtained by using BLASTx GenBank, comparing *Co. posadasii* gene and its ortholog.

[c] Largest ratio of expression between phases of the same isolate observed in the experiment. S = Silveira, C = C735, developmental phase 1,2, and 3.

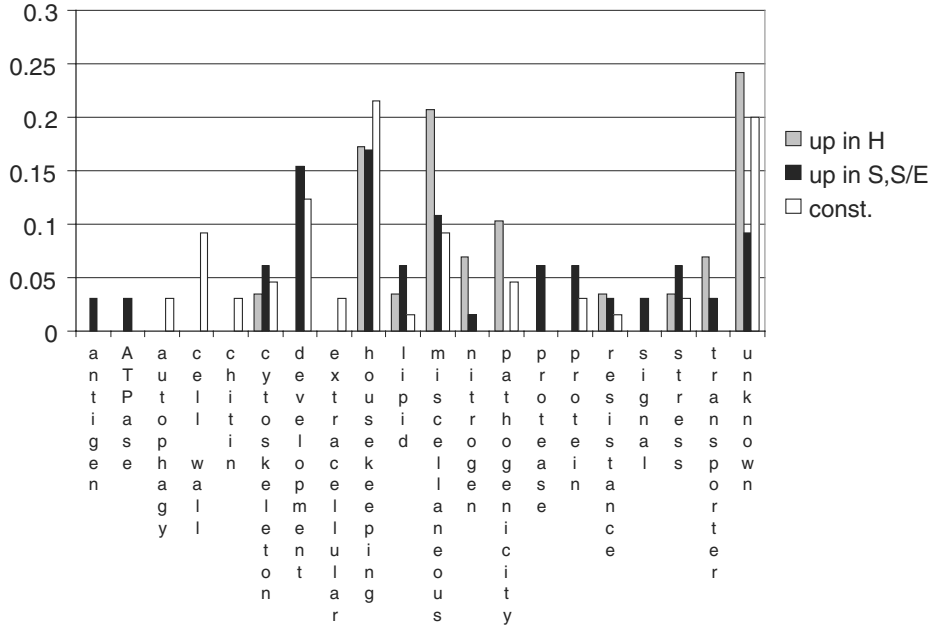

Fig. 5. Proportion of genes within three key expression patterns groups, found in assorted functional categories. Key expression patterns are *up in* H: Significantly upregulated in mycelial phase, *up in* S, S/E: Significantly upregulated in both parasitic phases, const: Not significantly differentially expressed between phases.

3.4. Between isolate variation in expression profile

More than half of the genes whose transcription differed between saprobic and pathogenic phases were significantly differentially expressed in only one of the isolates (Figs. 4 A and B). For many of these genes, different significance calls can be explained by a lack of statistical significance of the gene expression difference in one of the isolates when both show the same biological trend. However, statistically significant patterns of different gene expression between the two isolates were found for saprobic versus parasitic phases (27 genes between hyphae and presegmented spherules, 32 genes between hyphae and endosporulating spherules), and between the two parasitic phases (15 genes between presegmented and endosporulating spherules) (Fig. 7). For 10 genes, a differential pattern of gene expression was found for more than two phase comparisons (see Fig. 7 for examples). Although we do not have duplicate samples of each isolate at the parasitic phases, we did obtain a second biological sample for one genotype at the saprobic phase that was consistent with all of the above findings.

3.5. Quality control of microarray

Spotted 70-mers representing 835 of the 1000 ORFs for which oligomers had been arrayed passed our hybridization signal criterion, indicating expression of the corresponding genes in the investigated developmental phases.

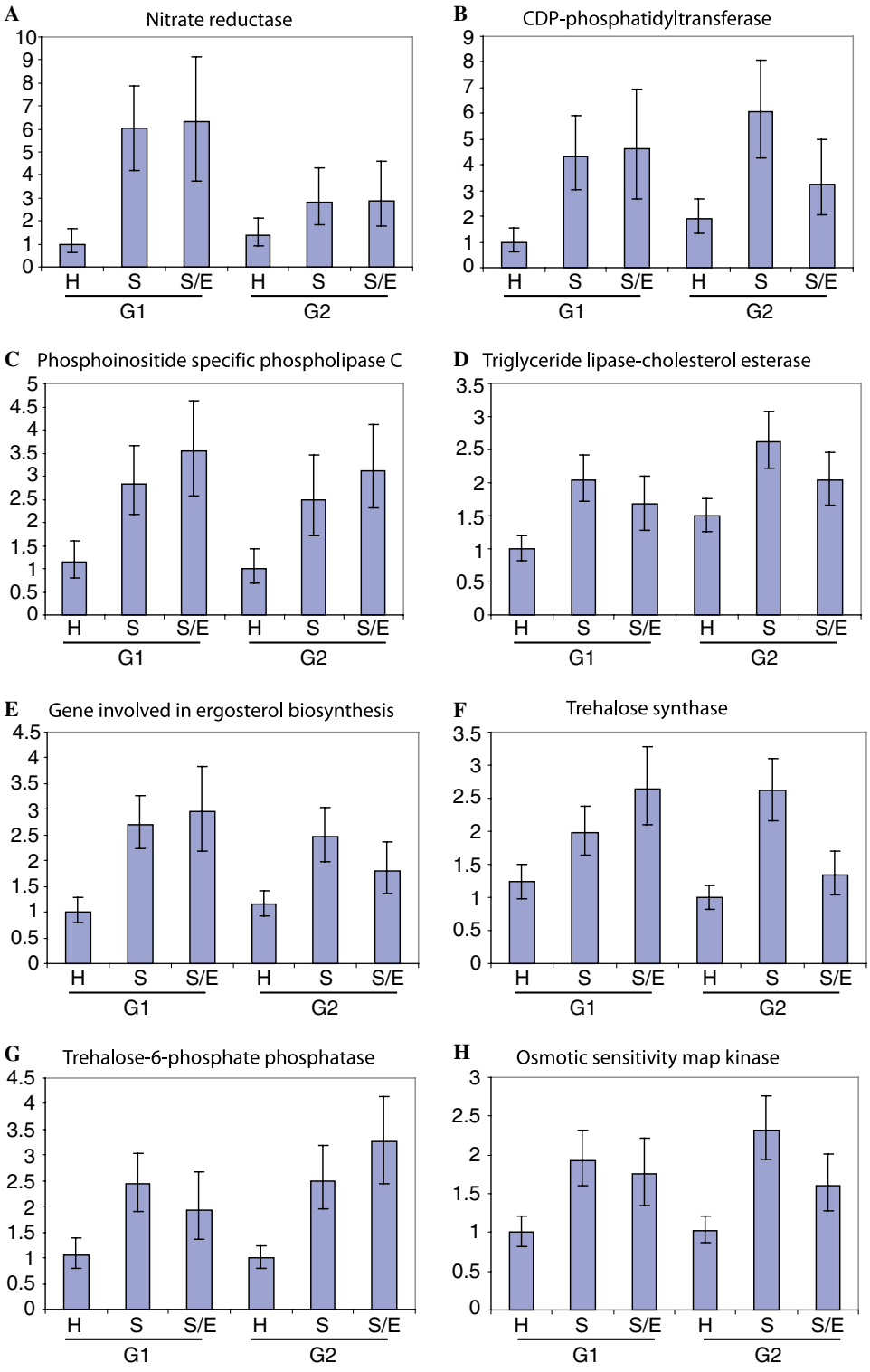

Fig. 6. Examples of genes with a significantly higher expression in parasitic phases (S and S/E) compared to the saprobic phase (H) in both C735 (G1) and Silveira (G2). Each bar represents the relative expression level obtained by BAGEL, with an asymmetric 95% credible interval indicated.

Furthermore, all replicate comparisons of the same nodes cluster together in the hierarchical clustering, except the direct dye swap replicates of the comparison (G2, H) vs. (G2, S/E), that are found close in the hierarchy, but do not cluster separately from the replicates of the (G2, H) vs. (G1, S) comparison (data not shown).

The four genes identified by Delgado et al. (2004) to be upregulated in the parasitic cycle of isolate C735 were also found to be significantly upregulated in endosporulating spherules as compared to mycelia (the two phases investigated in both studies) in isolate C735 of this study ($p < 0.05$). Furthermore, the highly elevated expression of

Table 2
Putative antigens

Gene Name	Annotation	Predicted MHC Class-II binding regions	Predicted localization
cole_103	Carboxypeptidase kex1	4	Extracellular
8x_1346_20g	Alpha-1,2 mannosyltransferase	1	Extracellular
cole_362	Endoglucanase/spore coat protein SP96	4	Extracellular
8x_595_14g	Endo-beta-1,3-1,4 glucanase	4	Extracellular
8x_1386_15g	Beta-mannosyltransferase	6	Extracellular
cole_381	IgE binding protein	3	Extracellular
8x_1304_22g	Unknown	6	Extracellular
cole_383	Allergen—Asp f9	3	Extracellular
cole_21	Alkaline phosphatase	5	Extracellular
8x_1147_26g	Expression library immunization antigen 1	7	GPI anchored
cole_268	Alpha-amylase AmyA	5	GPI anchored
cole_330	Chitinase 1	4	Extracellular
8x_476_15g	Putative secreted protein	1	Extracellular

the gene coding for a spherule outer wall glycoprotein (SOWgp) in cultures of presegmented spherules of isolate C735, reported by Hung et al., 2002 was confirmed in this study by a significant increase in expression between the mycelial and presegmented spherule phase in isolate C735 ($p < 0.001$), although in Silveira it appears to be highly expressed in all developmental phases. Finally, a constitutive expression previously observed between all three of the investigated phases for the genes glyceraldehyde 3-phosphate dehydrogenase (Hung et al., 2002) and ornithine decarboxylase (Guevara-Olvera et al., 2000) in isolate C735 was confirmed in this study. No significant difference in expression between the developmental states was found for these two genes by using microarrays.

3.6. Effect of sampling variation in the saprobic phase

Incorporating the biological replicate of the Silveira hyphal phase, and thus sampling variation due to growth and extraction, had a minor effect on the complement of genes detected as significantly differentially expressed. For instance, as reported above for the first biological sample, 182 genes were significantly upregulated between the hyphae and presegmented spherules for Silveira (Fig. 4A). When the biological replicate was included, 212 genes were found to be significantly upregulated, including 176 of the original 182; the remaining six showed the same biological trend, but the Bayesian probability had dropped from >0.95 to >0.92. The same pattern was found for the rest of the dataset; more genes were found to be significantly differential expressed in the different comparisons when the biological replicate was included, and the vast majority of genes found to be significantly differentially expressed in the first biological sample were also significant, or nearly so, when the biological replicate was added to the dataset.

4. Discussion

Despite the fundamental role the different morphological phases play in the course of coccidioidal infection, little is known about the differences in gene regulation between developmental phases. DNA microarray technology has the potential to illuminate studies of the molecular origin of phenotypic adaptation in human pathogens, particularly when analyzing multiple isolates from populations and species. We have made the first use of transcription profiling with *Coccidioides* and the first use of more than one isolate with a pathogenic fungus to develop a list of genes that are differentially expressed between developmental phases of two genetically distinct isolates of *Co. posadasii*. By combining the gene expression data with the predicted protein localization and binding sites for the genes, we identified a list of putative antigens of *Co. posadasii*. Investigating these genes further may lead to the discovery of new candidate antigens for the coccidioidal vaccine, as well as molecular targets for novel anti-fungal drugs (Boyle et al., 1994; Perfect, 2002). For our experimental design, the logistic regressions of the empirical power to detect differences in gene expression level indicated that we could identify most differences in gene expression above 1.5- to 2.2-fold. This method of analysis shows how our experiment was tailored to yield the greatest resolution for identifying differences in the samples of greatest interest. Our results show that comparison between isolates is an effective tool to reduce the pool of interesting genes to those that are common to more than one isolate.

One of the most interesting, significant results from this study is the observation of an increased expression of the nitrate reductase (*nir*) gene during parasitic phase growth in both isolates of *Co. posadasii*. This gene is likely to be an important virulence factor in *Coccidioides*, as previously has been suggested for *Mycobacterium* species (Weber et al., 2000). The *nir* gene is found in several species of bacteria and fungi, and is directly involved in the metabolic pathway of ammonia fermentation (Zhou et al., 2002). It allows fungi like *Fusarium oxysporum* and *Aspergillus nidulans* to grow under anoxic conditions suggesting that several fungi that are considered to be obligate aerobes could, in fact, be facultative anaerobes (Takasaki et al., 2004; Zhou et al., 2002). For example, *Mycobacterium tuberculosis* is a respiratory pathogen and is assumed to be an obligate aerobe challenged with an anaerobic environment

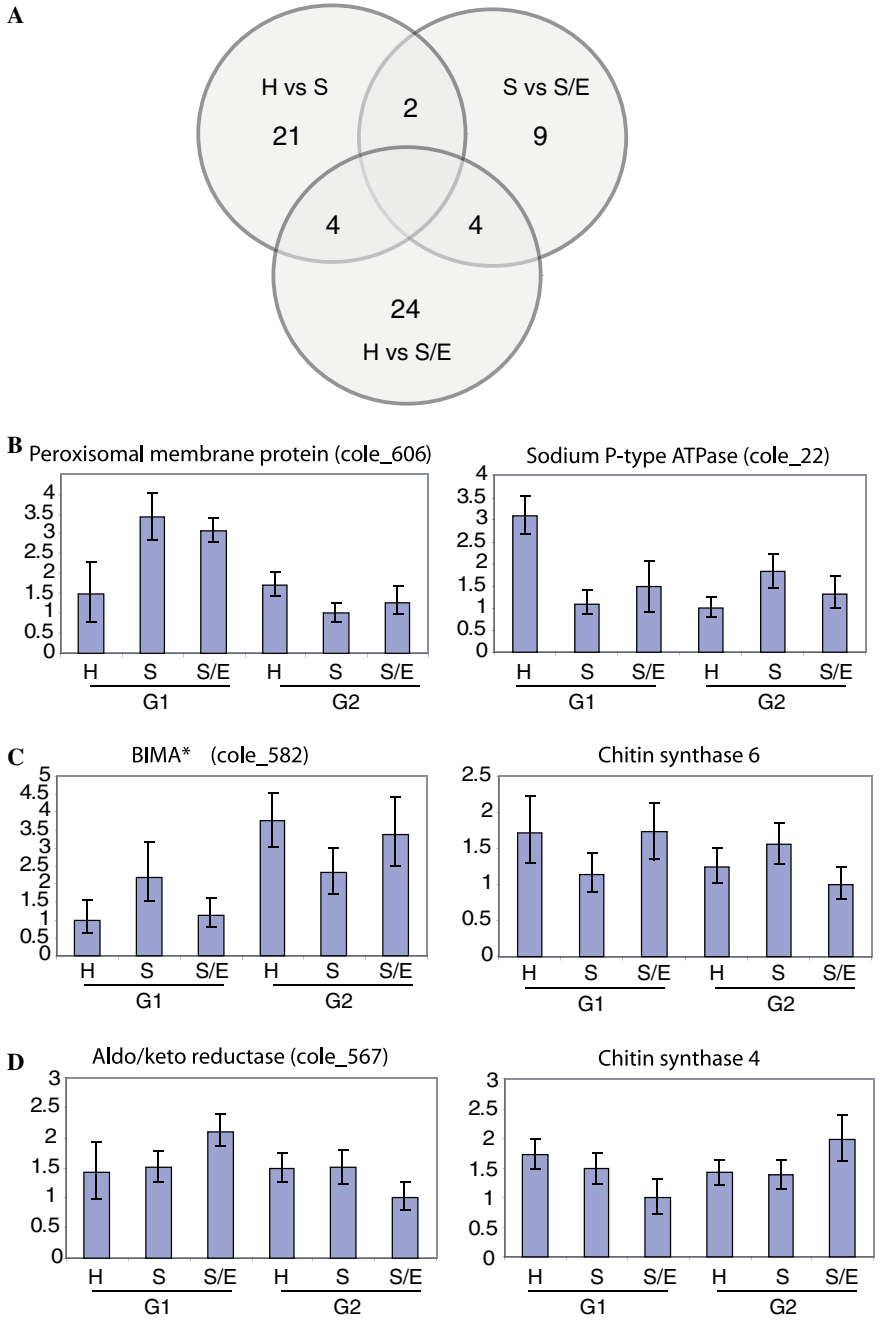

Fig. 7. (A) Venn diagram showing the number of genes exhibiting contradictory pattern of expression between the two genotypes. (B–D) Examples of genes differentially expressed between C735 (G1) and Silveira (G2) in more than one comparison of developmental phases. Each bar represents the relative expression level obtained by BAGEL, with an asymmetric 95% credible interval indicated. (B) Two examples of genes differentially expressed between developmental phase H and both S and S/E. (C) Between S and both H and S/E. (D) Between S/E and both H and S. *required for completion of mitosis in *A. nidulans*.

within abscesses and granulomas during infection. A mutant of *Mycobacterium bovis*, in which the nitrate reductase gene was deleted, had reduced virulence in a mouse model (Weber et al., 2000). Under aerobic conditions it grew as well as the wild type isolate. However, when the nitrate reductase deletion mutant was used to infect mice, the mice showed 100% survival well past 200 days. In contrast, when mice were infected with the wild type strain, they became ill and died after 90 days. In fact, mice infected with the mutant were able to clear the pathogen from their lungs, liver, and kidneys after 275 days (Fritz et al., 2002). Clearly, further investigation is warranted to investigate the potential role of *nir* in pathogenicity of *Coccidioides*.

Furthermore, several genes involved in the metabolism of lipids were up-regulated in the parasitic phase. Previous studies on the morphology of *Coccidiodes* isolates revealed that the parasitic phase spherules contain a higher proportion of lipid compared to mycelium or arthroconidia (Wheat et al., 1977); during parasitic phase growth, *Coccidioides* cells produce and shed a membranous, lipid-rich spherule outer wall

fraction (Cole et al., 1988b). Tarbet and Breslau (1953) showed that this spherule outer wall fraction is rich in complex lipids, which they identified as phospholipids, and more recent evidence has shown this fraction to be highly immunogenic (Cole et al., 1988a). Ergosterol is a prominent sterol found in fungal cell membranes; however it is not found in mammalian cells, making drugs such as amphotericin B or the triazoles (Vandenbossche et al., 1990) that target ergosterol in the cell membrane, effective anti-fungal agents. Knowledge that this group of genes is upregulated in the parasitic phase may help to refine the development of drugs targeting lipid metabolism in fungal pathogens.

A group of genes generally involved in the response to environmental stress was also upregulated in the parasitic phase of *Co. posadasii*. Trehalose is a disaccharide found in a wide variety of organisms including bacteria, plants, insects, and fungi (Gancedo and Flores, 2004), and in this study two enzymes involved in the synthesis of this disaccharide, trehalose synthase and trehalose-6-phosphate phosphatase, were up-regulated in spherules and endospores as compared to mycelia. Trehalose protects cells from environmental stresses such as heat, cold, desiccation, and oxidation, and the observed result is most likely due to thermal stress. The absence of trehalose in mammalian cells makes this pathway an attractive drug target. For example, a *Candida albicans* mutant in which trehalose phosphate phosphatase was disrupted was highly thermosensitive and failed to grow at 44 °C. Trehalose phosphate, which is highly toxic to fungi, accumulated within the cells of the mutant, and both the formation of hyphae and the ability of the fungus to infect mice were impaired (Zaragoza et al., 1998). Furthermore, a trehalose synthase mutant from *Cryptococcus neoformans* was found to have reduced infectivity in a mouse model; the pathogen was found to be cleared from the host within 3–7 days post-infection (Wills et al., 2002). Another gene involved in the response to environmental stresses is the osmotic sensitivity map kinase, which was found to be up-regulated in the parasitic phase cycle of *Co. posadasii*. This gene, which is highly conserved in yeasts and filamentous fungi, is up-regulated in response to high salt conditions. Upon activation, glycerol accumulates within the fungal cell, and this mechanism plays an essential role in maintaining water homeostasis. This hyperosmotic stress pathway is the target of phenylpyrrole fungicides, which are believed to exert their anti-fungal effects through the hyperactivation of the fungal signal transduction pathway (Kojima et al., 2004). In *N. crassa*, deletion of the osmotic sensitivity map kinase resulted in the inability of the fungus to grow under high salt conditions (4% NaCl), and the pyrrole fungicide Fludioxonil caused the conidia and hyphal cells to swell and burst (Zhang et al., 2002). Similar results were obtained with the fungal pathogen *Colletotrichum lagenarium* (Kojima et al., 2004). Furthermore, the osmotic sensitivity kinase has been proposed to be an important virulence factor in *Ca. albicans*, in which a deletion mutant was constructed and the resulting mutant was found to have reduced pathogenicity in a mouse model (Alonso-Monge et al., 1999, 2003). This pathway may be important to the virulence of *Coccidioides*, and is worth further examination.

It is noteworthy that the gene coding for the Woronin body major protein was found to be abundantly expressed in the saprobic phase compared to the parasitic phases. The Woronin body is a peroxisome-related organelle and functions to seal septal pores of filamentous ascomycetes in response to cellular damage (Jedd and Chua, 2000; Tey et al., 2005). The organelle has not been identified in ascomycetous yeasts, which do not develop septal pores (Yuan et al., 2003). Our results are consistent with the yeast results in that the Woronin body is expressed in *Co. posadasii* when the fungus is in the saprobic phase and producing septate hyphae, but is not expressed when the fungus is in the pathogenic phases and growing as a unicell.

A key result was that many genes showed differential expression between the two isolates. Approximately half of the genes significantly up- or down-regulated in one of the isolates show similar, significant differences in regulation in the other isolate. The half not showing significant differences in regulation in both isolates included genes showing the same pattern of expression, but not significantly so ($p < 0.05$), as well as genes with a significantly different expression pattern in the two isolates.

Among the genes for which differential expression apparently is due to inherent isolate differences is the spherule outer wall glycoprotein (*SOWgp*), which has been shown to be important in *Coccidioides* pathogenicity. The highly elevated expression of *SOWgp* in early parasitic phase cultures of isolate C735, reported by Hung et al. (2002), was confirmed in this study by a significant increase in expression in the presegmented spherule phase, as compared to mycelia ($p < 0.001$). However, no differential expression of *SOWgp* was found between the developmental phases in Silveira and the gene appears to be highly expressed in all phases of this isolate (data not shown). This finding was confirmed in repeated analyses of the original biological sample of Silveira mycelia, and with the biological replicate of Silveira mycelia. *SOWgp* is suggested to be important for pathogenicity of *Coccidioides* spp both by functioning as an adhesin and by modulating the hosts' immune response (Hung et al., 2000, 2002).

Even more striking are the differences between the isolates for other, less-well-studied, genes. Expression profiles between developmental phases for the two isolates were found to be significantly different and oppositely directed for 64 genes, of which 10 genes were found to be differentially expressed between isolates for more than two phase comparisons (Fig. 7). In light of these results, it is worth noting that it is the norm to use a single genotype in microarray studies that profile phase-specific gene expression in human pathogenic fungi, such as *Ca. albicans* (Lan et al., 2002), *Cr. neoformans* (Kraus et al., 2004), and *Histoplasma capsulatum* (Hwang et al., 2003). Studies of intraspecific variation gene expression in the yeast *Saccharomyces cerevisiae* (Fay et al., 2004; Townsend et al., 2003), the fruit fly *Drosophila melanogaster* (Drnevich et al., 2004), the teleost

fish *Fundulus heteroclitus* (Whitehead and Crawford, 2005), and in humans (Rockman and Wray, 2002) have all identified significant variation. The extent of variation identified has been dictated by the power of the study to detect significant small differences in gene expression, as prescribed by the number of replicates and samples examined (Townsend, 2004). Our results highlight the importance of understanding population variation before making general conclusions from a study of gene expression, particularly because the isolates examined originated from a single local population of *Co. posadasii*. The effects of strain-to-strain variation may be even more dramatic when individuals from divergent populations are examined.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2006.02.003.

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