Title

Genetic differences between Coccidioides spp. and closely related nonpathogenic Onygenales

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Identification and characterization of genes found in *Coccidioides* spp. but not nonpathogenic Onygenales

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Abstract

Coccidioides spp. are dimorphic, pathogenic fungi that can cause severe human and animal disease. Like the other primary fungal pathogens, infections of animals results in a morphologic transformation to a tissue phase, which in this case is known as a spherule. The sequencing and annotation of *Coccidioides* spp. and the genomes of several nonpathogenic Onygenales species allows comparisons that might provide clues about the *Coccidioides* spp. genes that might be involved in pathogenesis. This analysis is a gene by gene orthology comparison. Although there were few differences in the size of genes families in the *Coccidioides* spp.-specific group compared to the genes shared by *Coccidioides* spp. and nonpathogenic Onygenales, there were a number of differences in the characterization of the two types of genes. Many more *Coccidioides* spp.-specific genes are up-regulated expression in spherules. *Coccidioides* spp.-specific genes more often lacked functional annotation, were more often classified as orphan genes and had SNPs with stop codons or higher non-synonymous/ synonymous ratios. Review of individual genes in the *Coccidioides* spp.-specific group identified two genes in the Velvet family, a histidine kinase, two thioredoxin genes, a calmodulin gene and ureidoglycolate hydrolase. Velvet genes have been found to be important for mycelium differentiation to yeast in *Histoplasma capsulatum*. Hopefully, identification of these genes will be useful for pursuing potential *Coccidioides* spp. virulence genes in the future.

Keywords: Fungi, pathogenic fungi, *Coccidioides immitis*, *Coccidioides posadasii*, genomics, pathogenesis.

Introduction

Coccidioides immitis and Coccidioides *posadasii* are dimorphic fungi that are found in the soil in the desert regions of the American southwest, Mexico and South America (1-3). Both species can cause disease known as Valley Fever in normal people. The two species are closely related, morphologically indistinguishable and the major phenotypic difference between the two that is currently recognized is their geographic distribution; *C. immitis* is found primarily in California (including Baja California) and *C posadasii in* Arizona and other endemic areas (4, 5). The organism grows as mycelia in the soil and forms arthroconidia within the mycelium that are dispersed as the soil is disturbed by wind, construction or other events. If the arthroconidia are inhaled by susceptible hosts they undergo transformation into a spherical tissue form, known as a spherule. These structures divide internally to form as many as 100 endospores, which in turn can differentiate into spherules. Endospores can be released 96 hours after initial spherule formation, so the number of spherules can increase quickly. Infection with *Coccidioides* spp. causes symptomatic disease in 40-50% of immunocompetent hosts and in a substantial number of infections can be prolonged and/or severe. In some cases dissemination of the infection beyond the lung occurs. Some infections require life-long treatment and severe infections can be fatal.

Coccidioides spp. are a member of the Ascomycete Onygenales order which contains a number of other human pathogenic fungi, including *Histoplasma capsulatum*, *Paracoccidioides* spp., *Blastomyces dermatitidis* and the dermatophytes *Microsporum* spp. and *Trichopyton* spp.(6). In addition to these pathogens, several fungal species that are not human pathogens are also Onygenales (6, 7). One of these, *Uncinocarpus reesii*, forms arthroconidia within mycelia that are morphologically indistinguishable from *Coccidioides* spp. (8). *U. reesii* is found in the soil and has a worldwide distribution. It is keratinophilic and thermotolerant (9, 10). It utilizes

amino acids and peptides as well as carbohydrates for growth (11). *U. reesii* does not cause human disease but a close relative, *Chrysosporium zonatum*, has been reported to cause an infection in a single patient with chronic granulomatous disease (10). Despite the difference in lifestyle, *U. reesii* is closely related to *Coccidioides* spp. on a genetic basis (7, 8, 12).

The genome sequencing of multiple isolates of *Coccidioides* spp. and *U. reesii* has made it possible to identify genes that are present in *Coccidioides* spp. but not this close nonpathogenic relative. At least four other nonpathogenic species exist in the Onygenales (6). The genomes of these organisms, *Amauroascus mutatus*, *Amauroascus niger*, *Byssoonygenn creatinophilia*, *Chrysosporium queenslandicum* and *Onygena corvina* have also been sequenced and the size of gene families and the gain or loss of genes compared to *Coccidioides* spp. and *U. reesii* and other fungi (6). Whiston and Taylor found about 800 *Coccidioides* spp. genes that were not present in the nonpathogenic Onygenales. One distinctive feature of the *Coccidioides* spp.-specific genes was the increased frequency of over-expression in spherules (6).

Comparison of the of *Coccidioides* spp. genes not found in nonpathogenic Onygenales to those shared between *Coccidioides* spp. and nonpathogenic Onygenales might provide insights into genes that are required for pathogenesis of coccidioidomycosis. The purpose of the study is to identify these genes and analyze their characteristics.

Methods

Orthology

Initial orthololology searches were done using the orthology tool at FungiDB (http://fungidb.org/fungidb/showApplication.do). This tool compare twos sets of proteins by BLASTP and computes the percent match length. The thresholds for blast results were an e value $< 10^{-5}$ and a percent match length of $\geq 50\%$. Paralogs, orthologs and ortholog groups were found using OrthoMCL Pairs. Orthologs shared by *C. immitis* RS, H538.4 and *C. posadasii* Silvera and RMSCC 3488 were identified. Those genes were then compared to *U. reesii* using the same tool. Comparison to the predicted genes of non-pathogenic Onygenales,

Amauroascus mutatus, *Amauroascus niger*, *Byssoonygenn creatinophilia*, *Chrysosporium queenslandicum* and *Onygena corvina* was done using BLASTP, with a cut off e value of 10^{-6}. The gene predictions and annotations for these species were done by Jason Stajich, using data from Whiston and Taylor (6). All *Coccidioides* spp.-specific genes were blasted once against *U. reesii* to confirm there were no significant matches. The *C. immitis* R.S. gene ID notation is used for all gene designations.

Blast

BLASTP searching was done using Stand-alone BLAST from NCBI, using the default settings. Blast hits were identified using an upper limit e value of 10^{-6}. In addition to the searches mentioned above, searches against fungal proteins were done using a representative sample (3 million predicted proteins) of the fungal protein database downloaded from NCBI. Comparison of genes to short read archives (SRA) was done using ncbi-magicblast-1.3.0. The SRA archives used for this study were SRR3468018 and SRR1737468 from the study by Engelthaler et. al (13).

SNPS

C. posadasii Silvera coding region SNPs were identified by Engelthaler et. al (13). This species was chosen because C. immitis RS SNPs were not identified in this study. The data was downloaded from FungiDB.

Gene characterization

Other characteristics, such as the genetic location, ortholog group, number of orthologs, predicted signal peptides, predicted transmembrane domains, PFAM domains and relative level of expression in spherules and mycelia were downloaded from FungiDB. Up-regulation was defined by the criteria described by Whiston et. al., comparing mycelia to day 4 spherules OR a two fold increase in expression as defined by Viriyakosol et. al., comparing mycelia to day 2 spherules (14, 15).

Supplementary data

Supplemental Table 1 is an attached Excel table containing all the data used for these analyses.

Results

Coccidioides spp. genes that are shared by *C. immitis* RS and H538.4 and *C. posadasii* Silvera and RMSCC 3488 were chosen to identify genes that were common to each of the two species of *Coccidioides* spp. and exclude genes only found in one isolate or one species. 1101 genes are shared by these *Coccidioides* spp. but not found in *U. reesii* or the other non-pathogenic Onygenales. For brevity, these genes are referred to as *Coccidioides* spp.-specific genes and those that are common to the *Coccidioides* spp. and nonpathogenic Onygenales are referred to as common genes. The term *Coccidioides* spp.-specific does not imply that these genes do not have homologs in fungi other than nonpathogenic Onygenales and many do.

Table I

Comparison of *Coccidioides* spp.- specific and common genes

	Number (%)				
	Coccidioides spp.- specific		Common		P
Total	1101	11	6495	65	$<10^{-10}$
Median orthologous groups/gene	26		96		
Genes with blast match	707	64[a]	6192	95 [b]	$<10^{-10}$
Functional product description [c]	511	46	5241	81	$<10^{-10}$
PFAM domains	546	50	5666	87	$<10^{-10}$
Genes with stop codons in SNPS[d]	371	35	648	10	$<10^{-10}$
Non-syn SNP/syn SNP[e]>2	371	34	612	9	$<10^{-10}$
One or more transmembrane region	176	16	1244	19	N.S.
Signal peptide	159	14	850	13	N.S.
Up-regulated in spherules	496	45	1594	25	$<10^{-10}$

a: Compared to total number of *Coccidioides* spp.- specific genes; b: compared to total number of common genes; c: based on annotation; d; single nucleotide polymorphisms; e) Nonsynonymous/synonymous; P value calculated by proportions test in R.

Table 1 shows a comparison of the two sets of genes. Sixty five percent of all the *Coccidioides* spp. genes are common to nonpathogenic Onygenales and 11% are *Coccidioides* spp.-specific genes. The total is less than 100% because about one quarter of *C. immitis* RS genes do not

have orthologs in all three other *Coccidioides* spp. strains investigated. The predicted properties of proteins in the two groups are similar except for homology to other fungal proteins, the preferential expression in spherules and the amount of genetic variation. The median number of orthologs per gene, the percentage of genes with blast matches, PFAM domains or functional product descriptions are all higher in the common genes than the *Coccidioides* spp.-specific genes. As would be expected, common genes are homologous to wider diversity of fungi. Only 64% of the *Coccidioides* spp.– specific genes has a BLAST match to genes in other taxa, so 36% of them are completely lineage-specific or "orphan" genes. This value is higher than the overall percentage of orphan genes in *C. immitis*, which is about 18%. In contrast, only 5% of the common genes are orphans. The small number of orphan genes found in the common group are presumably homologous to genes in other fungi but with an e value that exceeds the cut off (10^{-6}). The fraction of *Coccidioides* spp.-specific genes with SNP stop codons or a non-synonymous/synonymous SNP ratio greater than two is higher than in common genes suggesting that the *Coccidioides* spp.-specific genes are evolving rapidly. The *Coccidioides* spp.-specific genes did not cluster on the genetic map. A larger proportion of the *Coccidioide*s spp.- specific genes are expressed at a higher level in spherules than mycelia, suggesting that this group of genes is enriched for genes associated with differentiation to spherules.

The distribution of some of the more common PFAM domains is shown in Table 2. There are some domains, such as the Armadillo-type fold, WD40 repeat or MFS_1 domains are somewhat less well represented in the *Coccidioides* spp.-specific group than in the common genes, indicating that these genes have been conserved in the non-pathogenic Onygenales as well as *Coccidioides* spp. These domains are found in a variety of proteins involved in many cell functions, including signal transduction, transcriptional regulation and cell cycle control, so it is not surprising that they are conserved in nonpathogens and well as *Coccidioides* spp. (16). The number of predicted domains in the *Coccidioides* spp.-specific group is relatively small, and few domains are found in multiple genes, so very few PFAM categories can be preferentially found in this group. Nevertheless, several domains appear slightly more common in *Coccidioides* spp.-

specific genes. This includes include protein kinase-like domains, HNH endonuclease, and oxireductases. However, none of these differences reaches statistical significance. There is also an enrichment of the phosphate transferase GO term GO:0016772 ($p = 2.06 \times 10^{-4}$) in the *Coccidioides* spp.-specific genes, which is further evidence of the enrichment of protein kinase genes. In contrast, a FunCat analysis shows an increased incidence of genes involved in secondary metabolism (Functional Category 01.20, $p = 1.20 \times 10^{-3}$) in the *Coccidioides* spp.-specific genes.

Table 2

The most common functional domains in *Coccidioides* spp.-specific and common genes

Description	Domain	Specific	Fraction	Common	Fraction	P
ARM repeat	SSF48371	0	0	45	0.008	0.01
WD-40	PF00400	0	0	27	0.005	0.002[a]
WD-40	SSF50978	0	0	24	0.004	
WD-40	PS50082	1	0.002	25	0.004	
MFS_1	PF07690	1	0.002	42	0.007	0.04
Phosphotransferase	PF01636	6	0.013	23	0.004	
HNH endonucleases	PF13391	6	0.013	5	0.001	
Protein kinase-like	PS50011	7	0.015	33	0.006	
Oxireductase	SSF51735	10	0.022	51	0.009	
Protein kinase-like	SSF56112	21	0.046	70	0.012	
alpha/beta-hydrolases	SSF53474	7	0.015	42	0.007	
NAD(P)-binding Rossmann-fold	SSF51735	10	0.022	51	0.009	
Others[b]		358		4851		
Total		454		5666		

Functional domains highlighted in yellow are statistically significantly more frequent in the common genes. a) calculated by aggregating all WD-40 domains; b) difference in fractions is less than 2.

Table 3

Comparison of up-regulated and unmodulated *Coccidioides* spp.–specific genes

	Up-regulated		Unmodulated		
	Number (%)				P
Total	493		605		
Orthologs/gene	15		40		
TM domains	65	13.18	109	17.93	0.036
Signal peptide	75	15.21	84	13.82	N.S.
No homologs outside *Coccidioides* (orphans)	180	36.51	214	35.37	N.S.
Product description "Hypothetical"	281	57.20	280	46.28	N.S.
Functional domains	214	43.03	332	54.60	2.5×10^{-4}

The up-regulated and unmodulated *Coccidioides* spp.–specific genes are listed in Supplemental Table 1 and a summary is shown in Table 3. The most significant differences between the two groups are the numbers of orthologs/gene and the fraction of genes with functional domains. The lower number of orthologs/gene in the up-regulated group suggests that these genes are more often found in a limited number of fungi. Review of the genes with functional domains (Supplemental Table 1) shows that protein kinase or protein kinase superfamily domains are the most common but they are present in the two groups at about the same frequency (3.1-3.5%).

Many of the *Coccidioides* spp.–specific genes are orphans and the proportion of orphans in up-regulated in spherules and constitutively expressed genes is the same. Orphan genes are well conserved within *Coccidioides* spp. By definition these genes are present in two *C. immitis* and two *C posadasii* isolates, and all of them are also found in one other isolate of each species that were selected for maximal divergence (13). 38% of the orphan genes have SNPs coding for stop codons, which is not different from all of the *Coccidioides* spp.-specific genes. However 59% of orphan genes contain SNPs with NS/S ratio greater than two, which is much higher than all of

the *Coccidioides* spp.-specific genes. This observation is consistent with hypothesis that orphan genes are not dispensable but are evolving rapidly. The origin of orphan genes is not clear. Although the function of these genes is difficult to determine, their conservation within *Coccidioides* spp. and up-regulation in spherules suggests that they may be important in differentiation into spherules.

A previous study has identified 26 genes that are up-regulated in *Coccidioides* spp. and *Histoplasma capsulatum* as the spherule or yeast differentiation occurs (17). Four of these are also found in the up-regulated *Coccidioides* spp.-unique group (Supplemental Table 1). One of the most interesting of these is CIMG_02628, an ARP2/3 complex subunit. This complex is composed of seven subunits that control nucleation of actin, which is important for cell wall remodeling and endocytosis (18). The transformation of mycelia to spherules requires extensive cellular remodeling, so the up-regulation of this gene seems reasonable. Two GMC/SPRK kinases are up-regulated *Coccidioides* spp.-specific genes; one of these, CIMG_02373, is also up-regulated in *H. capsulatum* yeast. A total of seven *Coccidioides* spp.-specific protein kinase genes are up-regulated in spherules, but more than 150 protein kinase genes of different types have been identified in *C. immitis*, and many are down-regulated in spherules, so the significance of this is not clear (15).

Analysis of individual *Coccidioides* spp.-specific genes

Because the number of *Coccidioides* spp.-specific genes was relatively small, individual genes of interest could be found by inspection. A number of these are shown in Table 4. Many other genes may be important but these are the ones that seem the most promising.

Table 4

Coccidioides spp.-specific genes of interest

Family	Product Description	Gene ID	Differentially regulated[a]	Known[b]
Velvet	Ryp 2/Velvet	CIMG_01530	Y	Y
	Vel C	CIMG_02440	N	N
Histidine kinase	NIK1	CIMG_09624	N	N
Thioredoxin	TrxA	CIMG_09126	N	N
	Thioredoxin	CIMG_09274	N	N
Calmodulin	Calmodulin	CIMG_04786	N	N
Ureidoglycolate hydrolase	UGH	CIMG_02178	Y	Y

a) Up-regulated in spherules; b) previously described in references (6, 19).

Velvet

The velvet family of genes are transcription factors than respond to external stimulate, such as light or stress, and trigger adaptive responses (20). Some of the responses include cell wall synthesis, trehalose synthesis, spore formation, secondary metabolism and a number of other functions in many fungi (21). Webster and Sil have reported that two members of the Velvet family, Ryp2 (also known as VosA) and Ryp3, are required for yeast formation at 37° in *H. capsulatum* and the pathogenesis of invasive histoplasmosis (22, 23). The *C. immitis* gene that Sil annotated as Ryp2 (CIMG_01530) does not have a homolog in nonpathogenic Onygenales. In contrast, Ryp3 is common to *Coccidioides* spp. and nonpathogenic Onygenales. Ryp2 and Ryp3 are up-regulated in *H. capsulatum* yeast compared to mycelia and expression of

CIMG_01530 is also up-regulated in spherules . Munoz has previously reported that Ryp2 is present in *C. immitis* and *C. posadasii* but not in *U. reesii* (19).

Another velvet family gene that is conserved in *C. immitis* and *C. posadasii* but not found in nonpathogenic Onygenales is VelC. The *Coccidioides* spp. VelC orthologs are truncated at the N-terminal region of the protein, but highly conserved with *Aspergillus fumigatus* and *Aspergillus nidulans* at the C-terminal Velvet domain. This member of the velvet family has not been as extensively studied as the others, but a recent study found it played an important role in sexual development in *A. nidulans*, despite the fact that studies in *A. fumigatus* of VelC mutants did not show a distinct phenotype (24). *Coccidioides* spp. contain genes required for mating, and recombination has been documented at the molecular level, but mating has not been observed. The function of VelC in *Coccidioides* spp. is unclear.

Histidine kinase

Histidine kinases are another group of proteins involved in responses to external stimuli (25). Some of the best know functions of histidine kinases include osmosensing, adaption to oxidants, conidiation, cell wall integrity and virulence. In some other primary pathogenic fungi, the Drk1 has been found to be required for the temperature dependent conversion from mycelia to yeast (26, 27). The Drk1 gene is found in both *Coccidioides* spp. and nonpathogenic Onygenales.

Goldberg described three histidine kinases in *C. immitis*, CIMG_04512, CIMG_05052 and CIMG_06539, which are all present in *U. reesii* (15) . However, there are more than ten histidine kinases in most filamentous fungi, so it is likely that more genes exist (28). In this analysis, we identified one other *Coccidioides* spp.-specific gene with a histidine kinase domain, which is not up-regulated in spherules. This gene (CIMG_09624) is almost completely identical in many isolates of *Coccidioides* spp. and very similar to histidine kinases in several other taxa (Fig 1). These data argue that this gene is very probably a highly conserved histidine kinase that may play an important role in the biology of *Coccidioides* spp.

Fig 1.

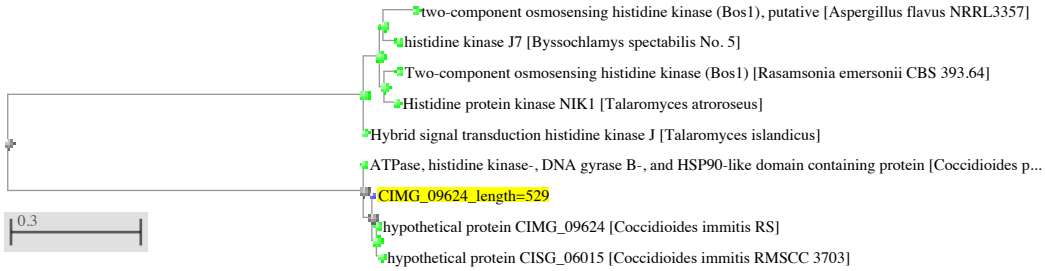

Fig.1 A phylogenetic tree obtained from Blastp analysis done comparing CIMG_09624 to all fungi.

Thioredoxin

Thioredoxin is an important regulatory enzyme that modifies target proteins by modifying their redox status (29). Most organisms have multiple thioredoxin genes with overlapping functions. The biological role of thioredoxin includes antioxidant functions that are probably particularly important for *Coccidioides* spp. when ingested by phagocytic cells. *C. immitis* is relatively resistant to oxidative stress and mice lacking an oxidative burst are not more susceptible than control mice to coccidioidomycosis (30). There are a total of three thioredoxin genes in *Coccidioides* spp., CIMG_09126, CIMG_09274 and CIMG_08211, none of which are up-regulated in spherules. Two of these, CIMG_09126 and CIMG_09274, are not found in nonpathogenic Onygenales. *U. reesii* does has have an ortholog of CIMG_08211. This difference in the number of thioredoxin genes may play a part in the ability of *Coccidioides* spp. to survive an oxidative burst and survive phagocytosis.

Calmodulin

Calmodulin is a calcium binding protein in the calcineurin pathway which is also important in regulating growth, responses to stress and virulence in a variety of fungi (31, 32). When calmodulin binds to calcium it activates calcineurin, which is critical for thermotolerance and pathogenicity. Calcineurin inhibitors inhibit the growth of a number of different fungi, including *C. immitis,* at 37°C (32, 33). There are three genes coding for calmodulin in *Coccidioides* spp.

and one of these, CIMG_04786 is a *Coccidioides* spp.- specific gene. *U. reesii* has only two genes coding for calmodulin. This difference in the number of genes may play a role in determining thermotolerance.

Ureidoglycolate hydrolase

Ureidoglycolate hydrolase (*UGH*), CIMG_02178 ,is a *Coccidioides spp*.-specific gene. Cole and his co-workers have studied the role of extracellular ammonia in spherule development (34). There is evidence that first generation spherules release endospores into an alkaline environment (34). One enzyme involved in ammonia production is ureidoglycolate hydrolase (*UGH*) and the expression of this gene is up-regulated in spherules compared to mycelia (14). The *C. posadasii* UGH deletion mutant is less virulent than the wildtype, even though the mutant grew normally as a mold in vitro (34). The urease/UGH double mutant, which makes very little ammonia, was much less virulent than wildtype. These data suggest that the UGH gene plays an important role in the pathogenesis of infections caused by *Coccidioides* spp.

Comparison to other studies

As part of a study of the genome of pathogenic Ajellomyces fungi compared to close non-pathogenic relatives Cuomo identified 46 *C. immitis* genes that were not found in *U. reesii* (19). The current analysis identified many more genes that were found in multiple *Coccidioides* spp. isolates but not nonpathogenic Onygenales but 24 of the genes were found in both studies. Cuomo also proposed 32 genes that were important for fungal pathogenesis. This analysis found only two homologs of these genes in Coccidioides spp. specific group: Ureidoglycolate hydrolase (*UGH*) and Ryp2. Whiston and Taylor have also compared the predicted proteins of *Coccidioides* spp. to nonpathogenic Onygenales (6). The number of *Coccidioid*es spp.-specific genes and the tendency of those genes to be over-expressed in spherules is similar in their study and this one.

Summary

This attempt to identify *Coccidioid*es spp. genes that are not found in nonpathogenic Onygenales is done to try to identify candidate pathogenesis genes. The strengths of the study include the overlap of these results with previous studies and the internal consistency of the data. This study used somewhat different methods than others but a significant number of *Coccidioides* spp.-specific genes were found in more than study. There are also a number of weaknesses. The identification of unique and common genes depends on the accuracy of the current annotation. Since *U. reesii* may not be annotated as well as *C. immitis* and *C. posadasii*, some genes might be classified as unique in error. Furthermore, a weakness of all these types of studies is the possibility that genes have evolved to perform new functions, so the absence of a given homolog in an organism does not imply the absence of that biological process. In addition, in the instances where a number of genes are found (such as thioredoxin and calmodulin) it is not clear what to expect from the result of the absence of one or two of them.

Many of the unique genes are not only divergent from non-pathogenic Onygenales, but lack homologs in other fungal taxa. The number of orphan common genes in the *Coccidioides* spp.-specific group is 46% compared to 5% in the common group. However, the orphan genes are found in at least four isolates of each species and two other distantly related isolates.

A common theme of the genes that are *Coccidioides* spp.-specific is their role in responding to environmental changes. Genes in the Velvet family, histidine kinases, calmodulin and thioredoxin are all important for responses to stress. In *Histoplasma capsulatum*, velvet genes are required for the mycelium to yeast transformation. In addition, *Coccidioides* spp.-specific genes tended to be up-regulated in spherules more often than *Coccidioides* spp. shared with nonpathogenic Onygenales, suggesting that more *Coccidioides* spp.- specific genes are involved in, or a consequence of, spherule development. Because many of the up-regulated *Coccidioides* spp.-specific genes are orphan genes their role in mycelium to spherule transformation is intriguing even though their function is unknown. Hopefully this data will suggest genes that

would be good candidates for further investigation of the molecular mechanisms of *Coccidioides* spp. pathogenicity.

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