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Unraveling the Molecular Basis of Temperature-Dependent Genetic Regulation in *Penicillium marneffei*

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***Penicillium marneffei* is an opportunistic fungal pathogen endemic in Southeast Asia, causing lethal systemic infections in immunocompromised patients. *P. marneffei* grows in a mycelial form at the ambient temperature of 25°C and transitions to a yeast form at 37°C. The ability to alternate between the mycelial and yeast forms at different temperatures, namely, thermal dimorphism, has long been considered critical for the pathogenicity of *P. marneffei*, yet the underlying genetic mechanisms remain elusive. Here we employed high-throughput sequencing to unravel global transcriptional profiles of *P. marneffei* PM1 grown at 25 and 37°C. Among ~11,000 protein-coding genes, 1,447 were overexpressed and 1,414 were underexpressed at 37°C. Counterintuitively, heat-responsive genes, predicted in *P. marneffei* through sequence comparison, did not tend to be overexpressed at 37°C. These results suggest that *P. marneffei* may take a distinct strategy of genetic regulation at the elevated temperature; the current knowledge concerning fungal heat response, based on studies of model fungal organisms, may not be applicable to *P. marneffei*. Our results further showed that the tandem repeat sequences (TRSs) are overrepresented in coding regions of *P. marneffei* genes, and TRS-containing genes tend to be overexpressed at 37°C. Furthermore, genomic sequences and expression data were integrated to characterize gene clusters, multigene families, and species-specific genes of *P. marneffei*. In sum, we present an integrated analysis and a comprehensive resource toward a better understanding of temperature-dependent genetic regulation in *P. marneffei*.**

With over one million new infections every year and latent infections worldwide growing to the tens of millions, dimorphic fungal infection presents many significant public health challenges to date (1–3). As the only known dimorphic species in the *Penicilliium* genus, *Penicillium marneffei* (recently renamed *Talaromyces marneffei*) is of particular concern. *P. marneffei* primarily infects immunocompromised individuals, causing lethal systemic infection, or penicilliosis (4–6). Over the last 20 years, there has been a marked increase in the number of penicilliosis cases, with a concurrent rise in immunosuppression due to the global spread of HIV infection (7–15). Penicilliosis is the third most prevalent opportunistic infection among HIV patients in Southeast Asia (16). Thus, infection by *P. marneffei* has become an AIDS-defining illness (6, 17).

P. marneffei, *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Coccidioides immitis*, *Paracoccidioides brasiliensis*, and *Sporothrix schenckii*, are collectively regarded as thermally dimorphic fungi, which undergo temperature-dependent transition between two distinct growth forms: mycelia and yeast. For instance, *P. marneffei* grows vegetatively as mycelia at 25°C, showing the typical multinuclear mold morphology; at 37°C, it undergoes the phase transition with concomitant coupling of nuclear and cellular division to form uninucleate, single-celled yeasts. The mycelium-to-yeast transition is considered to be a requisite for pathogenesis of *P. marneffei*, as the yeast cell is the *in vivo* form that capably evades the host immune system (18, 19). These features well place *P. marneffei* as a model experimental system for investigation of fungal growth processes and their contribution to pathogenicity (19).

Early dimorphic fungal studies were limited to morphological examination or clinical isolates and the cellular events accompanying the phase transition processes. Only in the past decade have studies focused on the molecular mechanisms of phase transition with the application of novel genetic approaches in *P. marneffei* (20–22). To date, more than 40 *P. marneffei* genes have been cloned or experimentally characterized (23–27). However, except for several transcriptional regulators and signal transduction factors, most of these genes are involved in vegetative growth and asexual differentiation (28). Thus, very little is known about genes responsible for phase-specific growth.

In a previous publication (29), we briefly announced the sequencing of the genome of *P. marneffei* strain PM1, without providing in-depth genomic and transcriptomic analyses. In the present study, we use comparative genomic and transcriptomic approaches to systematically characterize *P. marneffei* genomic sequences and global gene expression. We refine the annotation of protein-coding genes and then use high-throughput mRNA sequencing (RNA-seq) to measure gene expression in mycelia at 25°C and yeast cells at 37°C. Through comparative genomics between *P. marneffei* and several model fungal species, we gain new insights into the evolutionary history of the *P. marneffei* genome. Our transcriptomic analysis suggests the existence of uncharacter-

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ized regulatory pathways that might be essential for thermal adaptation in *P. marneffei*.

MATERIALS AND METHODS

DNA preparation, sequencing, and assembly. Genomic DNA was prepared from the arthroconidia of *P. marneffei* PM1 grown at 37°C. A single colony of the fungus grown on Sabouraud's dextrose agar (SDA) at 37°C was inoculated into yeast peptone broth and incubated in a shaker for 3 days. Cells were cooled in ice for 10 min, harvested by centrifugation at 2,000 × *g* for 10 min, washed twice, and resuspended in ice-cold 50 mM EDTA buffer (pH 7.5). Novozyme 234 (20 mg/ml) was added, and the mixture was incubated at 37°C for 1 h followed by digestion in a mixture of 1 mg/ml proteinase K, 1% *N*-lauroylsarcosine, and 0.5 M EDTA (pH 9.5) at 50°C for 2 h. Genomic DNA was then extracted by phenol and phenol-chloroform and finally precipitated and washed in ethanol. After digestion with RNase A, a second ethanol precipitation was followed by washing with 70% ethanol, and then the DNA was air dried and dissolved in 500 μl of Tris-EDTA (TE [pH 8.0]) (30). Two genomic DNA libraries were made in pUC18 carrying insert sizes from 2.0 to 3.0 kb and from 7.5 to 8.0 kb, respectively. DNA inserts were prepared by physical shearing using the sonication method (31). The genome sequence was assembled from deep whole-genome shotgun (WGS) coverage obtained by paired-end sequencing from a variety of clone types (i.e., all inserts were sequenced from both ends to generate paired reads). A total of 190.3 Mb of sequence data, which is equivalent to ~6.6× coverage of the genome, was generated by random shotgun sequencing. The Phred/Phrap/Consed (http://www.phrap.org/) package was used for base calling, contig assembly, and quality assessment (32, 33). Contigs were ordered into supercontigs (also known as scaffolds) by using the scaffold building program Bambus (http://www.cbcb.umd.edu/software/bambus/) (34).

RNA-seq analysis. At the beginning of the experiment, conidia of strain PM1 were inoculated in SDA plates and cultured at 25 and 37°C for a week. The germinated cells were transferred into new SDA plates every week for 2 weeks to establish stable colonies in either the mycelial or yeast growth form. One week before the extraction of total RNA, in order to obtain fresh cells, the homogenous cells were cultured in new SDA plates. The total RNAs were extracted from each condition for two independent biological replicates using the E.Z.N.A. fungal RNA kit (Omega Bio-Tek). The effect of global transcriptional amplification or suppression might lead to erroneous interpretation of gene expression experiments (35). To control for this effect, we adjusted the total RNA concentration according to the DNA content before the standard poly(A)$^+$ RNA-seq was performed. DNA was quantified using Qubit 2.0 fluorometer (Life Technologies, Grand Island, NY). We obtained ~13 million 90-bp paired-end reads for each sample. The reads were trimmed basing on their qualities by FastQC and fastx-trimmer. Filtered reads were mapped to the annotated genomes with Tophat v2.0.2 (36). For each sample, ~2.0 Gb of reads was mapped, representing ~100× coverage of *P. marneffei* transcriptome. Cufflinks v2.0.2 (36) was used to calculate the fragments per kilobase of exon per million fragments mapped (FPKM) (37) and the confidence internals of the estimation for each gene. For differential expression analysis, mapped reads were counted using SAMMate (http://sammate.sourceforge.net/) (38). Differentially expressed genes were detected by jointly using three R packages: edgeR (39), DESeq (40), and baySeq (41), which all are available at the Bioconductor open source bioinformatics software repository (http://bioconductor.org/). Genes with a false discovery rate (FDR) of <0.05 as reported by all three packages were considered to be differentially expressed.

Gene prediction and identification of orthologs. *Ab initio* gene predictions were performed using FGENESH (SoftBerry, Mount Kisco, NY). The original prediction was manually refined with assistance from GenomeScan (http://genes.mit.edu/genomescan.html), another gene prediction program that combines sequence similarity and exon-intron composition. The putative ortholog pairs were predicted by using InParanoid (http://inparanoid.sbc.su.se/) (42). Syntenies were identified using ADHoRe (http://bioinformatics.psb.ugent.be/software/details/ADHoRe) (43), with an r^2 cutoff of 0.8, maximum gap size of 35 genes, and minimum number of pairs of 3. The ADHoRe results were filtered such that the maximum probability for a segment to be generated by chance was <0.01. Gene families in each genome were identified by using NCBI BLASTClust (with 30% of identical residues and aligned over at least 80% of their lengths).

IFA staining with Mp1p-specific antibodies. *P. marneffei* was first cultured on SDA plates at 37 and 25°C for 7 days, respectively. Culture supernatants were obtained by inoculating fungal cells from plates into RPMI 1640 and shaken at 37 and 25°C for 5 days. Tissues were collected by centrifugation at 5,000 rpm for 20 min. The fungal mycelium and yeast cells were washed twice, resuspended in phosphate-buffered saline (PBS), and placed on wells of Teflon-coated slides. The immobilized cells were fixed for 10 min with prechilled fixed buffer containing acetone (70% [wt/vol]) and methanol (30% [vol/vol]) and stored at 70°C until they were used. The slides were incubated with the monoclonal antibodies (MAbs) against Mp1p at a concentration of 10 μg/ml in a humid chamber for 60 min at 37°C. The MAb was prepared by a procedure described previously (44, 45). Briefly, BALB/c mice were immunized intradermally with 50 mg of purified Mp1p emulsified with Freund's adjuvant at 10-day intervals for 8 weeks followed by intravenously administered boosters for 3 days before fusion. Antibodies specific for *P. marneffei* in the hybridoma cell culture supernatant were screened by indirect enzyme-linked immunosorbent assay (ELISA) using Mp1p and indirect fluorescent antibody (IFA) staining using slides with immobilized fungal cells killed in 3.7% formalin solution overnight at 4°C. The isotypes of the MAbs were determined using a commercially available mouse MAb isotyping kit (Zymed Laboratories, Carlsbad, CA). MAbs were purified from ascitic fluid by ammonium sulfate precipitation and were conjugated with biotin using the EZLink Sulfo-NHS-biotinylation kit following the manufacturer's instructions (Pierce Biotechnology, Rockford, IL). After the slides were washed with PBS containing 0.1% (vol/vol) Tween 20, fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Zymed Laboratories) was added and the slides were incubated for 40 min at 37°C. After several washes, a 0.25% (wt/vol) solution of Evans blue in PBS was added for counterstaining; fluorescence was determined by fluorescent laser confocal microscopy with a Leica TCS SP2 AOBS microscope.

GO analysis. The orthologs of *P. marneffei* genes in *Aspergillus fumigatus*, *Aspergillus nidulans*, *Candida albicans*, *Neurospora crassa*, or *Saccharomyces cerevisiae* were identified, and then the Gene Ontology (GO) terms of orthologs of *P. marneffei* in all five model fungal species were taken together. Nonredundant terms were assigned to corresponding *P. marneffei* genes. In GO term enrichment tests, we grouped GO terms in the resulting lists into major classes based on the hierarchical structure between GO terms using REVIGO (46). Significance of functional enrichment of GO terms in differentially expressed gene sets was assessed using the chi-square test. All resulting *P* values were adjusted for multiple hypothesis testing using the method of Storey and Tibshirani (47), limiting the FDR to 0.05.

Identification of coding tandem repeats. The intragenic tandem repeats in the *P. marneffei* genome and other fungal genomes were identified by a previously described method (48) based on EMBOSS ETANDEM (49). The threshold score was set to 20. All genes were scanned for long repeats (>40 nucleotides [nt]) or short repeats (3 to 39 nt). A sequence was considered to be an intragenic repeat if it meet two conditions: (i) repeat conservation was at least 85%, and (ii) the numbers of repeats were at least 20 for trinucleotide repeats, 16 for repeats between 4 and 10 nt, 10 for repeats between 11 and 39 nt, and 3 for repeats of at least 40 nt.

Data sources for model fungal organisms. The genome and protein sequences of model fungal organisms were obtained from the following sources: AspGD (50) for *A. fumigatus* and *A. nidulans*, the *Candida* Genome Database (CGD) (51) for *C. albicans*, the *Neurospora crassa* Database (http://www.broadinstitute.org/annotation/genome/neurospora) for *N. crassa*, and the *Saccharomyces* Genome Database (SGD) (52) for *S.*

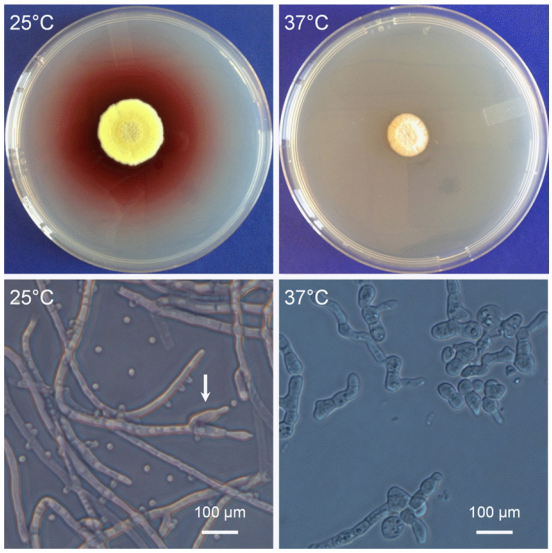

FIG 1 Temperature-dependent dimorphism in *P. marneffei*. (Top panels) One-week *P. marneffei* grown on SDA plates. Soluble red pigments were produced and diffused into the agar at 25°C; a yeast-type colony formed with decreased production of pigments at 37°C. (Bottom panels) ×400 microscopic views of long mycelia, conidia, and conidiophore (white arrow) at 25°C and fission yeast-like cells (arthroconidia) at 37°C.

cerevisiae. The phenotypic information of single mutants, used to identify heat-responsive genes, was collected from AspGD (50) for *A. fumigatus* and *A. nidulans*, and PhenomeBLAST for *S. cerevisiae*.

Data accession number. Expression data have been submitted to the GEO database under accession number GSE48898.

RESULTS

Under controlled laboratory conditions, *P. marneffei* PM1 displayed two temperature-dependent morphologies: the mycelial form at 25°C and the yeast form at 37°C (Fig. 1). We took a systematic approach, coupling genome sequence and transcriptome analyses, to investigate the genetic regulation of thermal dimorphism in *P. marneffei*. The rest of the article is organized as follows. First we outline the basic features of the genome sequence of *P. marneffei* PM1 and present the global results of RNA-seq analysis defining its transcriptome. Subsequently, we describe the annotation of gene functions and detect *P. marneffei* genes expressed differentially between mycelial cells at 25°C and yeast cells at 37°C, emphasizing the expression of heat-responsive genes. Finally, we integrate the genome sequencing and expression data to describe gene clusters, genes with tandem repeat sequences in the coding region, and multigene families and species-specific genes in *P. marneffei*.

Genome sequences and transcriptome profile of *P. marneffei*. The draft genome of *P. marneffei* PM1 was obtained using the whole-genome shotgun (WGS) approach (29). A total of 311,029 WGS reads of an average length of 690 bp were assembled into 2,780 contigs, which were subsequently ordered into 273 supercontigs (Table 1). The assembly produces a 6.6× coverage of the *P. marneffei* genome of an estimated size of ~31 million bp. The genome size of *P. marneffei* is comparable to those of *Aspergillus fumigatus*, *A. nidulans*, and *Penicillium chrysogenum* (29.4, 30.5, and 32.2 Mb, respectively) (53–55). A total of 10,060 protein-coding genes were initially predicted *in silico*. The genic regions occupy 62.1% of the total genome sequences, while the protein-coding sequences occupy 51.2%. The average gene density is one gene per 2.8 kb, and the average gene length is 1.75 kb. A total of 28,180 introns were found in 91% of *P. marneffei* genes. The size of introns ranges from 15 to 1,617 bp, with a mean of 111 bp. The telomere tandem repeat TTAGGG, along with telomere-associated helicases, was identified at the end of 16 contigs, suggesting 8 chromosomes in *P. marneffei*, as in *A. fumigatus* and *A. nidulans*.

We precultured isogenic *P. marneffei* PM1 on solid media at 25 and 37°C. Mycelial and yeast cells were collected for RNA-seq analysis. Over 13 million paired-end reads 90 bp long for each sample were generated with two biological replicates. After short-read mapping and transcript calling, the expressed transcripts were obtained and compared against the predicted genes. Through the comparison, 982 novel transcripts, overlapping with at least a 50-bp open reading frame (ORF), were identified. Accordingly, the total number of predicted protein-coding genes was updated to 11,042. Among these genes, 26.34% (2,908 genes) are expressed into more than one isoform through alternative splicing, making a total of 15,567 unique transcripts. The expression level was estimated at the whole-gene level in fragments per kilobase of exon per million fragments mapped (FPKM) (37). In mycelial cells at 25°C, 95.2% of genes (10,508) and 89.8% of transcripts (13,993) were expressed (FPKM > 0.05); in yeast cells at 37°C, 97.2% of genes (10,730) and 91.6% of transcripts (14,256) were expressed. Jointly, 98.5% of genes (10,882) and 95.6% of transcripts (14,886) were expressed at either temperature.

To identify *P. marneffei* genes expressed at a significantly different level between mycelial cells at 25°C and yeast cells at 37°C, we conducted differential expression analysis with RNA-seq data. Here we followed the general practice of assuming that all samples placed into the analysis platform contain similar amounts of total RNA (cf. 35). We required that the differentially expressed (DE) genes be those detected as significant by all three commonly used algorithms—namely, edgeR (39), DESeq (40), and baySeq (41)—at the level of a false discovery rate (FDR) of <0.05 simultaneously. Using such conservative criteria, a total of 2,861 DE genes were identified, including 1,447 overexpressed and 1,414 underexpressed in yeast cells at 37°C, compared with mycelial cells at 25°C (see Table S1 in the supplemental material).

There are 32 *P. marneffei* genes whose expressions were previ-

TABLE 1 General assembly features of the *P. marneffei* genome

Feature	Value
Coverage	6.6
Estimated genome size, Mb	~31
Assembly length (excluding gaps), Mb	28.98
Total contig length, Mb	28.89
Scaffolds, no.	273
Contigs, no.	2,780
GC genome, %	47
Predicted protein-coding genes, no.	10,060
Portion of coding, %	62
GC coding, %	50
Mean gene length, bp	1,753
Mean intergenic distance, bp	1,051
Mean exon length, bp	380
Mean intron length, bp	111
tRNAs, no.	110

TABLE 2 Comparison of gene expression results from previously published studies with those from our RNA-seq study

Gene	Function	Previously reported result		RNA-seq result in this study					
				25°C		37°C			
		Expression change at 37°C	Reference	Replicate 1	Replicate 2	Replicate 1	Replicate 2	Expression change at 37°C	Replication
abaA	Transcriptional regulator	↓	27	163.3	170.3	20.1	18.4	↓	Yes
actA	Actin	—	79	1313.2	1,350.2	688.8	890.4	—	Yes
acuD	Isocitrate lyase	↑	80	198.5	486.8	217.3	225.0	—	No
areA	Transcriptional activator	—	81	30.1	44.3	37.1	50.6	—	Yes
brlA	Transcription factor	↓	82	1,407.6	1,340.8	5.1	4.2	↓	Yes
cflA	Rho family GTPase	—	83	153.3	149.1	140.0	122.6	—	Yes
cflB	Rho family GTPase	—	84	110.4	116.0	45.2	73.0	—	Yes
cpeA	Catalase-peroxidase	—	85	595.3	934.8	329.8	105.9	—	Yes
drkA	Two-component histidine kinase	—	86	72.5	73.5	40.1	46.8	—	Yes
gasA	G-protein complex a subunit	—	87	71.5	64.3	100.1	69.5	—	Yes
gasC	G-protein complex a subunit	↓	23	102.3	108.9	39.2	41.8	↓	Yes
gpdA	Glyceraldehyde-3-phosphate dehydrogenase	↓	88	3,280.2	3,146.5	2,701.4	2,209.3	—	No
hsp30	Heat shock protein	↑	89	1,059.1	764.4	2,645.7	3,081.7	—	No
hsp70	Heat shock protein	↑	90	2,401.0	2,503.0	1,259.7	1,757.3	—	No
mp1	Antigenic mannoprotein	↑	91	1.6	3.1	1,551.1	493.6	↑	Yes
mplp6	Antigenic mannoprotein	↑	92	0.0	0.0	1,081.4	177.5	↑	Yes
myoB	Type II myosin	—	93	32.8	18.0	17.8	18.6	—	Yes
pakA	p21-activated kinase	—	94	46.2	47.5	42.4	46.5	—	Yes
pakB	p21-activated kinase	↓	26	68.1	82.3	44.9	38.1	—	No
hhk1	Histidine kinase	—	95	11.8	10.3	9.0	15.4	—	Yes
rsr1	RAS small monomeric GTPase	↑	96	22.1	18.3	39.8	17.4	—	No
rasA	Ras GTPase	—	97	45.9	38.7	24.8	20.7	—	Yes
rfxA	Transcriptional regulator	—	21	82.3	68.1	52.1	65.7	—	Yes
sakA	Stress-activated protein kinase	—	98	201.3	215.8	165.6	103.0	—	Yes
skn7	Stress response transcription factor	—	99	72.0	80.6	47.5	35.8	—	Yes
slnA	Two-component histidine kinase	—	86	11.1	10.9	23.6	19.8	—	Yes
sodA	Cu, Zn superoxide dismutase	—	100	409.1	674.6	500.5	785.9	—	Yes
stlA	Control of mating and yeast-hyphal transitions	—	101	43.3	55.9	30.6	49.9	—	Yes
stuA	APSES transcription factor	↓	24	256.8	403.5	25.7	49.9	↓	Yes
tbpA	RNA polymerase I and III transcription factor	—	25	379.0	674.6	507.5	532.7	—	Yes
tupA	Transcriptional repressor	—	102	76.2	49.9	41.8	38.9	—	Yes

[a] ↑, expression is higher at 37°C than at 25°C; ↓, expression is lower at 37°C than at 25°C; —, no difference in expression.

ously measured mostly by using microarrays at 25 and 37°C or in mycelial and yeast cells (Table 2). We made a comparison of gene expression measures between our RNA-seq results and these previously published results. We found that 26 of the 32 genes showed a consistent pattern in the direction of expression change, suggesting a high reproducibility of DE analysis across different measurement methods and *P. marneffei* strains.

Biological functions of 14 *P. marneffei* genes in the morphogenetic control for conidial germination, hyphal growth, asexual development, and yeast morphogenesis so far have been elucidated (as reviewed in reference 28). Among six genes known to play a role at 25°C exclusively, three (*brlA*, *gasC*, and *stuA*) were found to be expressed more abundantly at 25°C than at 37°C (Fig. 2). Among eight genes functioning at both 25°C and 37°C, *abaA* is required in asexual development and yeast growth and is known to be overexpressed at 25°C (27). Our RNA-seq data showed that *abaA* is indeed expressed significantly higher at 25°C, while the other seven genes are expressed at similar levels at the two temperatures (Fig. 2).

Functional annotation of *P. marneffei* genes. To predict the function of *P. marneffei* genes, we employed *in silico* annotation through comparative genomics across fungal species. For each *P. marneffei* gene, functional annotation information (e.g., GO terms) of orthologous genes in other model fungal species were transferred. The model fungal species included *Aspergillus fumigatus*, *Aspergillus nidulans*, *Candida albicans*, *Neurospora crassa*, and *Saccharomyces cerevisiae*. A total of 8,161 protein-coding genes of *P. marneffei* were annotated with at least one GO term. We found that 261 GO terms are overrepresented in *P. marneffei* genes overexpressed at 37°C (FDR < 0.05) (see Table S2 in the supplemental material). These terms include "RNA-directed DNA polymerase activity," "misfolded RNA binding," "group I intron splicing," and "snoRNA localization" (Table 3). On the other hand, we found that 453 GO terms are overrepresented in *P. marneffei* genes underexpressed at 37°C (i.e., overexpressed at 25°C) (FDR $<$ 0.05) (see Table S3 in the supplemental material). These terms include "α-1,4-glucan synthase activity," "response to light stimulus," and "secondary metabolic processes."

Expression analysis of heat-responsive genes in *P. marneffei*. To study the expression regulation of the heat response, we compiled a list of 52 heat-responsive genes whose function has been characterized using gene knockout experiments in *A. fumigatus*, *A. nidulans*, or *S. cerevisiae*. Using this collection of genes, we identified 168 potential heat-responsive genes in *P. marneffei*. Our RNA-seq results showed that only 6 (3.6%) *P. marneffei* heat-responsive genes are overexpressed at 37°C, which is significantly fewer than expected (FDR < 0.05). We also collected another list of genes related to a broad sense of heat shock response identified by using two-dimensional (2D) fluorescence difference gel electrophoresis in *A. fumigatus* (56). Our RNA-seq results showed that *P. marneffei* orthologs of most of these genes are not expressed differentially at 25 and 37°C (see Table S4 in the supplemental material). Furthermore, none of the heat shock proteins and heat shock factors in *P. marneffei* were found to be overexpressed at 37°C (Table 4).

Next we started from a list of 12 GO terms concerning heat

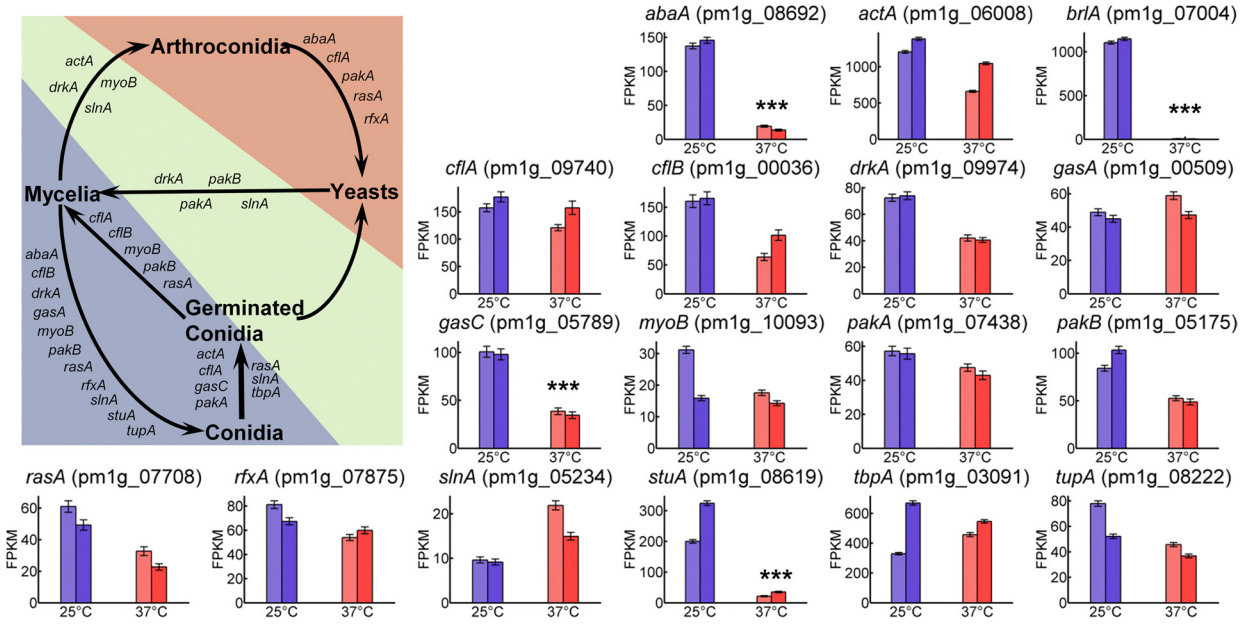

FIG 2 Expression levels of morphogenesis genes of *P. marneffei*. The left panel depicts the life cycle and dimorphic switch of *P. marneffei*. The blue region (bottom left) represents processes in the mycelium form, the red region (top right) represents processes in the yeast form, and the green region (middle) represents processes involved in the dimorphic switching. The right panel shows FPKM values of gene expression measured at 25 and 37°C. Each bar represents an independent replicate. ***, significant difference in levels of expression of the gene at 25 versus 37°C (see Materials and Methods for details).

response and found none of these GO terms are enriched in genes overexpressed at 37°C (Table 5). Notably, GO:0036168 (with the term "filamentous growth of a population of unicellular organisms in response to heat") is underrepresented in genes overexpressed at 37°C and overrepresented in genes underexpressed at 37°C. Among 370 *P. marneffei* genes whose annotation is linked with any of the 12 heat response GO terms, 16 are overexpressed at 37°C, which is significantly fewer than 52 genes underexpressed at 37°C ($P < 0.001$, Fisher's exact test).

Taken together, these results suggest the limited value of using the existing gene annotations available in other fungal species in annotating *P. marneffei* genes, especially for genes that might play a role in heat response. The reason for this might be that *P. marneffei* has an uncharacterized regulatory network and signaling pathways in response to heat, which differ considerably from those currently known in other fungal species.

Gene clusters in *P. marneffei* and other filamentous fungi. To identify gene clusters conserved across species, we compared the genome sequences of *P. marneffei* with those of *A. nidulans*, *A. fumigatus*, and *N. crassa*. We revealed extensive regions of con-

TABLE 3 Top representatives of GO terms enriched in *P. marneffei* genes upregulated or downregulated at 37°C

	GO term in genes with responses at 37°C	
Function	Upregulated	Downregulated
Cell components	GO:0030686—90S preribosome GO:0005730—nucleolus	GO:0000329—fungal-type vacuole membrane GO:0005887—integral to plasma membrane GO:0051286—cell tip GO:0016020—membrane GO:0032153—cell division site
Biological processes	GO:0003964—RNA-directed DNA polymerase activity GO:0008026—ATP-dependent helicase activity GO:0000293—ferric-chelate reductase activity GO:0034336—misfolded RNA binding	GO:0004497—monooxygenase activity GO:0000036—acyl carrier activity GO:0033201—α-1,4-glucan synthase activity GO:0004553—hydrolase activity, hydrolyzing *O*-glycosyl compounds
Molecular	GO:0015116—sulfate transmembrane transporter activity GO:0042254—ribosome biogenesis GO:0000372—group I intron splicing GO:0048254—snoRNA localization GO:0040010—positive regulation of growth rate GO:0006675—mannosyl-inositol phosphorylceramide metabolic process	GO:0016208—AMP binding GO:0045461—sterigmatocystin biosynthetic process GO:0009416—response to light stimulus GO:0019748—secondary metabolic process GO:0055114—oxidation-reduction process GO:0015707—nitrite transport

TABLE 4 Gene expression of putative heat shock proteins and heat shock factors in *P. marneffei*

Gene ID	Homolog(s)	FPKM at:				Expression change at 37°C
		25°C		37°C		
		Replicate 1	Replicate 2	Replicate 1	Replicate 2	
pm1g_01498	*hsp12* of *S. cerevisiae*	1,594.7	1,015.9	17.5	12.1	↓
pm1g_02064	*hsp60* of *A. fumigatus*	262.2	215.8	199.9	476.7	—
pm1g_03635	*ssc70* (mitochondrial *hsp70*) of *A. fumigatus*	392.4	389.7	591.2	1,225.2	—
pm1g_05682	*hsp30* of *A. fumigatus*	988.1	825.0	2,520.4	3,467.3	—
pm1g_06129	*hsp104* of *S. cerevisiae*	159.9	120.9	150.2	152.3	—
pm1g_06571	*hsp78* of *A. fumigatus*	58.7	38.7	64.3	87.0	—
pm1g_08006	*hsp70* of *A. fumigatus*	2,224.6	2,401.0	1,175.3	1,977.2	—
pm1g_10441	*hsp90* of *A. fumigatus*	591.2	591.2	629.3	941.3	—
pm1g_10905	*hsp88* of *A. fumigatus*	285.0	339.1	301.3	406.3	—
pm1g_01128	*aha1* (*hsp82* cochaperone) of *S. cerevisiae*	138.1	156.6	221.9	283.0	—
pm1g_05408	*hsf1* of *A. fumigatus*	51.0	55.5	52.1	37.1	—
pm1g_05918	*sti1* (*hsp90* cochaperone) of *S. cerevisiae*	138.1	150.2	167.9	316.4	—
pm1g_08054	*sis1* (*hsp40* cochaperone) of *S. cerevisiae*	232.9	232.9	120.1	100.1	↓
pm1g_08850	*hlj1* (*hsp40* cochaperone) of *S. cerevisiae*	42.4	43.3	37.9	22.8	—

served syntenies, as well as a considerable extent of genome reorganization between these fungal species. For example, there are 1,340 regions containing four or more genes colinear between *P. marneffei* and *A. nidulans* and 1,273 between *P. marneffei* and *A. fumigatus*. Totals of 3,188 and 3,716 *P. marneffei* genes were found to be involved in the two cases, respectively. The largest syntenic cluster contains 27 gene pairs, appearing in *P. marneffei* and *A. nidulans*.

Melanin is made in various pathogenic fungi and has been implicated in the pathogenesis of a number of fungal infections (57). Melanin may play a role in inhibiting cytokine production in the host cells (58) and apoptosis in macrophages (59). In *A. fumigatus*, the two kinds of melanin, dihydroxynaphthalene-melanin (DHN-melanin) and pyomelanin (60–63), are generated. Six genes related to the biosynthesis of DHN-melanin were clustered together (60, 61) and genetically regulated by the expression of *brlA* (64). Our previous study revealed the existence of this gene cluster in *P. marneffei* (22). RNA-seq data from this study showed that all six genes in the cluster are significantly downregulated at 37°C (Fig. 3).

Furthermore, we identified the pyomelanin biosynthesis gene cluster, which is also conserved between *P. marneffei* and *A. fumigatus*. Pyomelanin biosynthesis pathway is tightly linked with the pathway of tyrosine degradation: two genes, *hppD* and *hmgX*, known to be specifically involved in the pyomelanin biosynthesis pathway, are colocated in the same cluster with the other four genes in the tyrosine degradation pathway (62, 63) (Fig. 3). The identified cluster in *P. marneffei* spans a 17-kb genomic region in a single contig. Half of the genes in the cluster were found to be expressed significantly higher at 37°C. The *P. marneffei* cluster, 6 kb longer than that of *A. fumigatus*, contains a gene between *hmgR* and *maiA*, whose ortholog in *A. fumigatus* is relocated. The ex-

TABLE 5 Enrichment analysis of GO terms related to heat response for *P. marneffei* genes

GO no.	GO term	No. of genes with GO term (FDR)[a]		
		All genes (n = 8,161)	Genes overexpressed at 37°C (n = 1,447)	Genes underexpressed at 37°C (n = 1,414)
GO:0009408	Response to heat	148	1 (**1.96×10^{-4}**)	22 (0.292)
GO:0031072	Heat shock protein binding	44	3 (0.173)	4 (0.294)
GO:0031990	mRNA export from nucleus in response to heat stress	68	0 (**6.52×10^{-3}**)	7 (0.279)
GO:0034605	Cellular response to heat	259	11 (**5.33×10^{-4}**)	45 (**8.52×10^{-2}**)
GO:0036165	Invasive growth in response to heat	3	0 (0.194)	2 (**3.13×10^{-2}**)
GO:0036168	Filamentous growth of a population of unicellular organisms in response to heat	175	7 (**3.21×10^{-3}**)	34 (**3.57×10^{-2}**)
GO:0061408	Positive regulation of transcription from RNA polymerase II promoter in response to heat stress	13	1 (0.206)	1 (0.293)
GO:0070370	Cellular heat acclimation	83	0 (**3.32×10^{-3}**)	13 (0.313)
GO:0070414	Trehalose metabolism in response to heat stress	2	0 (0.187)	0 (0.260)
GO:1900432	Negative regulation of filamentous growth of a population of unicellular organisms in response to heat	62	0 (**8.51×10^{-3}**)	5 (0.301)
GO:1900433	Positive regulation of filamentous growth of a population of unicellular organisms in response to heat	60	1 (**2.08×10^{-2}**)	11 (0.268)
GO:2000728	Regulation of mRNA export from nucleus in response to heat stress	5	0 (0.192)	0 (0.296)

[a] Significant values where FDR is <0.05 are in boldface.

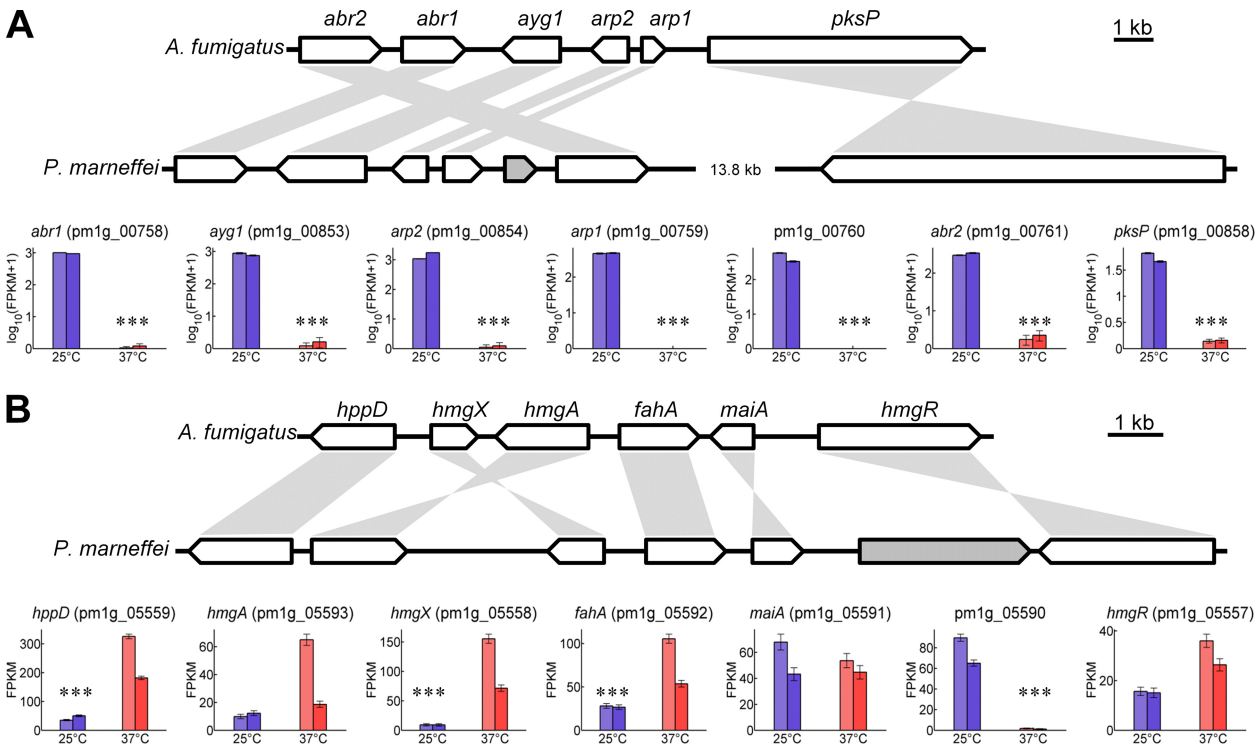

FIG 3 Gene cluster organization and expression of two melanin biosynthesis clusters in *P. marneffei*. The gene cluster of DHN-melanin biosynthesis (A) and the gene cluster of tyrosine degradation (B) in *A. fumigatus* and *P. marneffei* are shown. The expression level (FPKM) is shown in the log scale for DHN-melanin genes and the linear scale for tyrosine degradation genes. ***, significant difference in levels of expression of the gene at 25 versus 37°C (see Materials and Methods for details).

pression of the gene, with potential catalytic activity, is downregulated at 37°C.

A. fumigatus DHN-melanin biosynthesis genes are highly expressed at 37°C, suggesting that DHN-melanin is implicated in *A. fumigatus* infection (65–67). Our results showed that *P. marneffei* produces less DHN-melanin and more pyomelanin at 37°C, suggesting that pyomelanin, instead of DHN-melanin, plays a role in the pathogenicity of *P. marneffei*.

Abundant TRSs in coding regions of *P. marneffei* genes. Tandem repeat sequences (TRSs) are two or more adjacent copies of the same sequence of nucleotides resulting from tandem duplication events. TRSs located in the coding region of genes produce repeated units of amino acids in the genes' protein products. These protein polymorphisms may create quantitative alterations in fungal phenotypes, such as adhesion, flocculation, and biofilm formation (48). We compared the relative abundance of coding TRSs between *P. marneffei* and three other filamentous fungi (Table 6). *P. marneffei* shows the highest *B*/*G* ratio, where *B* represents the bases in coding TRSs and *G* represents the genome size. That is to say, *P. marneffei* accommodates more coding TRSs per unit of genome than other fungal species under consideration. We hypothesized that the presence of abundant coding TRSs in *P. marneffei* genes might allow the fungal cells to "disguise" themselves in order to evade the host immune system's defenses. If true, the expression of TRS-containing genes should be upregulated at 37°C. Indeed, among 66 genes that contain coding TRSs, 15 were found to be overexpressed at 37°C (see Table S5 in the supplemental material). The number is significantly higher than expected ($P < 0.01$, Fisher's exact test). No specific DNA sequence motif was identified in 5′ regulatory regions of these genes, suggesting that the upregulated expression of these genes may not be controlled by a master transcription factor.

TABLE 6 TRSs in coding regions of the fungal genomes

Species	TRSs in coding region (kb) (*B*)	Genome size (Mb) (*G*)	*B*/*G* ratio
P. marneffei	23.8	30	0.79
A. fumigatus	12.7	28	0.45
A. nidulans	16.8	30	0.56
N. crassa	22.1	40	0.55

Multigene families and species-specific genes in *P. marneffei*. Among protein-coding genes of *P. marneffei*, 2,283 of them belong to 665 multigene families (see Materials and Methods for details about gene family classification). The most expanded gene families include the following: the MFS multidrug transporter family (34 genes), the short-chain dehydrogenase or reductase family (31 genes), the hexose transporter family (27 genes), the pepsin-type protease family (24 genes), and the major facilitator superfamily (23 genes). Notably, the expansion of the MP1 domain protein (Mp1p, a cell wall antigen) has been proposed to be implicated in *P. marneffei* pathogenesis (68). Twelve genes containing the MP1 domain have been identified in *P. marneffei*; in contrast to that, only two MP1 domain genes have been identified in *A. fumigatus* (53, 69–71). Among these 12 genes, 7 were found to be overexpressed and one underexpressed at 37°C (Fig. 4A).

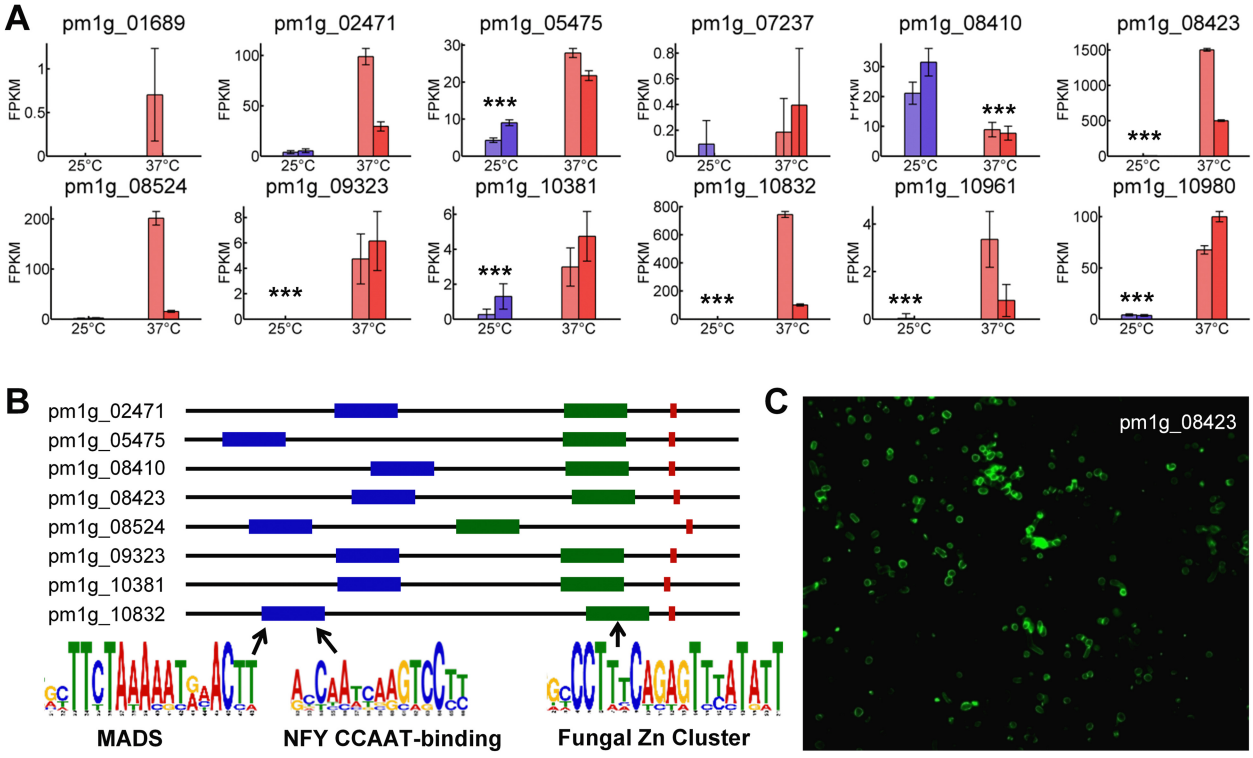

FIG 4 Expression levels of *P. marneffei* MP1 domain genes and their regulatory regions. (A) Expression levels of 12 MP1 domain genes. ***, significant difference in levels of expression of the gene at 25 versus 37°C (see Materials and Methods for details). (B) Putative transcription factor binding sites in 5′ regulatory regions. The positions of binding motifs are indicated with the blue and green bars and labeled with the subtype name of transcription factors: (i) MADS, (ii) NFY CCAAT binding, and (iii) fungal Zn cluster. The position of the TATA box is indicated by the red bar. (C) Indirect immunofluorescent staining with antibodies to Mp1p (pm1g_08423) at 37°C.

Using a motif-discovering tool, MEME (72), we identified conserved DNA sequence motifs in the 5′ regulatory regions of these MP1 domain genes (Fig. 4B). These motifs are the putative binding sites of subtypes of transcription factors: (i) MADS, (ii) NFY CCAAT binding, and (iii) fungal Zn cluster (73). These results indicate that these MP1 domain genes are likely to be regulated by shared transcription factors. Next, to confirm the cellular localization of Mp1p protein products, we performed the indirect fluorescent antibody (IFA) staining assay with antibodies for Mp1p (pm1g_08423) (see Materials and Methods). The results confirm that the Mp1p gene is expressed at 37°C and that the gene product is located on the cell wall of yeast cells (Fig. 4C). No expression and protein product were detected at 25°C. Furthermore, we used between-supercontig sequence comparison to search for conserved gene order. No evidence for large-genomic-segment duplication in *P. marneffei* was identified. Compared with *S. cerevisiae*, the *P. marneffei* genome contains relatively fewer recently duplicated gene pairs, as indicated by the small nonsynonymous-to-synonymous substitution ratio (K_a/K_s) between two copies of duplicate genes ($P < 0.01$, Fisher's exact test) (74). These differences can be explained by the whole-genome duplication in *S. cerevisiae* (75).

Finally, we identified 1,009 *P. marneffei* genes whose protein sequences do not share any sequence similarity with other protein sequences in the NCBI nonredundant database (BLASTP E value cutoff of 1e−10). Among these orphan genes, 107 are not expressed at either 25 or 37°C. In the remaining 902 expressed ones, 117 (13.0%) are overexpressed at 37°C (FDR > 0.05) and only 63 (7.0%) are underexpressed at 37°C (FDR < 0.05). Thus, *P. marneffei*-specific genes are not likely to be overexpressed but are less likely to underexpressed in yeast cells at 37°C.

DISCUSSION

P. marneffei is an emerging model system for investigating fungal growth processes and their contribution to pathogenicity (28). To understand the genetics of *P. marneffei*, especially in response to elevated temperature, we jointly used genome sequences and transcriptomic profiles to unravel the molecular basis of genetic regulation in *P. marneffei*. Previous studies of *P. marneffei* transcriptome were based on microarrays (76, 77). The adaptation of the RNA-seq approach, which measures gene expression in a much higher dynamic range, allowed us to produce an unbiased, high-resolution-expression measurement of *P. marneffei* genes for the first time.

Globally, we identified 1,414 and 1,447 *P. marneffei* protein-coding genes as being up- or downregulated at 37°C, respectively, compared with at 25°C. That is, more than a quarter of genes in the *P. marneffei* genome are expressed at different levels at two temperatures. This figure is likely to be a lower bound of the estimation as we adapted highly stringent statistical criteria for asserting DE genes. Next the myriad of genes presented a great challenge to gene function annotation. We leveraged comparative genomics across fungal species and detected a variety of annotation features over- or underrepresented in heat-responsive genes in *P. marneffei*. Some of these discoveries seemed to fit our expectations well.

For example, the family of MP1 domain protein (Mp1p) is expanded significantly in *P. marneffei*, and the majority of members of this family are overexpressed in yeast cells at 37°C. A second example is the expansion of the Mp1p protein family in *P. marneffei*, suggesting that duplicate genes confer novel phenotypic changes and facilitate organismal adaptation. Moreover, we hypothesized that *P. marneffei* genes whose coding region contains TRSs are likely to be overexpressed at 37°C, because products of these genes may play a role in helping the fungus escape the attack of immune system of the host cell. Indeed, *P. marneffei* has more abundant coding TRSs in its genes, and these genes are more likely to be overexpressed at 37°C. In addition, genes involved in biosynthesis of pyomelanin were found to be upregulated at 37°C, while genes involved in biosynthesis of DHN-melanin were found to be downregulated at 37°C, which suggests conditional regulation for specific melanin biosynthesis pathways.

Many genes and their expression patterns negated our expectations. For example, we expected that genes whose annotation contains GO terms related to heat should tend to be expressed higher than other genes at 37°C. However, we found that significantly fewer "heat-related" genes in *P. marneffei* were upregulated at 37°C. We also expected that *P. marneffei*-specific genes are likely to be overexpressed at 37°C. This is because species-specific genes are likely to define the unique biology feature of the species and play a role in response to environmental changes (47). However, we found that *P. marneffei*-specific genes were not enriched in genes overexpressed at 37°C.

These unexpected results suggest that functional annotation of *P. marneffei* genes through mapping of annotation from orthologous genes remains unreliable. Given that *P. marneffei* is a unique species in its genus, it is possible that our current knowledge about genetic regulation of heat response is inapplicable to *P. marneffei*. Furthermore, we might have to update our view with regard to the link between thermal dimorphism and pathogenesis in *P. marneffei*. The new idea is that steadily low expression of heat-responsive genes may confer virulence to *P. marneffei* grown at the temperature of 37°C in mammalian host cells. In *Phytophthora sojae*, for example, the virulence of the plant-pathogenic fungus is attained by the silencing instead of activation of a gene (78). We hypothesize that the pathogenicity of *P. marneffei* is associated with the suppressed expression of heat-responsive genes at 37°C, and the phase transition of *P. marneffei* may result from gene silencing. In other words, the yeast form is an impaired form of the filamentous form.

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