Extracellular Vesicle-Mediated RNA Release in Histoplasma capsulatum

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ABSTRACT  Eukaryotic cells, including fungi, release extracellular vesicles (EVs). These lipid bilayered compartments play essential roles in cellular communication and pathogenesis. EV composition is complex and includes proteins, glycans, pigments, and mRNA. The mRNAs with putative roles in pathogenesis have been described in EVs produced by fungi. Here we describe the RNA content in EVs produced by the G186AR and G217B strains of Histoplasma capsulatum, an important human-pathogenic fungal pathogen. A total of 124 mRNAs were identified in both strains. In this set of RNA classes, 93 transcripts were enriched in EVs from the G217B strain, whereas 31 were enriched in EVs produced by the G186AR strain. This result suggests that there are important strain-specific properties in the mRNA composition of fungal EVs. We also identified short fragments (25 to 40 nucleotides in length) that were strain specific, with a greater number identified in EVs produced by the G217B strain. Remarkably, the highly enriched processes were stress responses and translation. Half of these fragments aligned to the reverse strand of the transcript, suggesting the occurrence of microRNA (miRNA)-like molecules in fungal EVs. We also compared the transcriptome profiles of H. capsulatum with the RNA composition of EVs, and no correlation was observed. Taking the results together, our study provided information about the RNA molecules present in H. capsulatum EVs and about the differences in composition between the strains. In addition, we found no correlation between the most highly expressed transcripts in the cell and their presence in the EVs, reinforcing the idea that the RNAs were directed to the EVs by a regulated mechanism.

IMPORTANCE Extracellular vesicles (EVs) play important roles in cellular communication and pathogenesis. The RNA molecules in EVs have been implicated in a variety of processes. EV-associated RNA classes have recently been described in pathogenic fungi; however, only a few reports of studies describing the RNAs in fungal EVs are available. Improved knowledge of EV-associated RNA will contribute to the understanding of their role during infection. In this study, we described the RNA content in EVs produced by two isolates of Histoplasma capsulatum. Our results add this important pathogen to the current short list of fungal species with the ability to use EVs for the extracellular release of RNA.

KEYWORDS Histoplasma capsulatum, RNA, extracellular vesicles

Histoplasma capsulatum is a major human fungal pathogen on the global stage that causes disease in both immunocompetent and immunocompromised individuals, albeit the risk for severe disease increases with compromised immunity (e.g., in patients with HIV infection or cancer as well as in individuals receiving steroids or tumor necrosis

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factor alpha (TNF-α) blockers). In the United States, it is the most common cause of fungal pneumonia (1). *H. capsulatum* is of particular concern in certain developing regions (2), especially in Latin American countries, including Brazil (3, 4), Guatemala (5), and French Guiana, where it is considered the “first cause of AIDS-related death” (6).

Despite its clear importance, enormous gaps exist in our understanding of the pathogenesis of histoplasmosis, the disease caused by *H. capsulatum*. An interesting facet of the biology of *H. capsulatum* is its ability to release extracellular vesicles (EVs) (7, 8).

EVs are bilayered lipid structures released by remarkably diverse cells across all kingdoms (9). We have demonstrated that EVs are present in both ascomycetes and basidiomycetes (7, 10–14). This observation implies that mechanisms for EV production and release are truly ancient, as they appear to predate the divergence of these branches 0.5–1.0 billion years ago. Fungal EVs can carry biologically active proteins, carbohydrates, lipids, pigments and nucleic acids (15, 16), many of which are constituents of the fungal cell wall and diverse others are associated with stress response and pathogenesis.

EV-mediated transport of fungal RNA was recently shown in both commensal and opportunistic fungi. EV RNA molecules, mostly smaller than 250 nucleotides (nt), were identified in *Cryptococcus neoformans, Paracoccidioides brasiliensis, Candida albicans, Saccharomyces cerevisiae*, and *Malassezia sympodialis* (17, 18). Since *H. capsulatum* packages diverse compounds within EVs, we postulated that it too would use these compartments to export RNA. In this study, the EV-associated RNA components were characterized in two different isolates of *H. capsulatum*. As described in other fungi, *H. capsulatum* EVs carry both mRNAs and noncoding RNAs (ncRNAs). In addition, proteomic data allowed the identification of 139 RNA-binding proteins (RBPs) in the EVs, suggesting that proteins involved in RNA metabolism might play an important role in cell communication through the EVs. Our results add this important pathogen to the list of fungal species with the ability to use EVs for the extracellular release of RNA.

**RESULTS**

*Histoplasma capsulatum* EVs contain RNA. We characterized the RNA molecules contained in EVs isolated from culture supernatant samples of *H. capsulatum* strains G186AR and G217B. These strains belong to distinct clades, and G217B has been shown to be more virulent than G186AR in experimental models (19, 20). The best-known difference between these two strains is that G217B lacks alpha-1,3-glucan on the yeast form cell wall (19, 20).

The reads obtained from the mRNA libraries (reads of >200 nt) were aligned with each strain-specific genome available at the NCBI (G186AR ABBS02 and G217B ABBT01). For data validation, we considered only sequences with expression values of transcripts per million (TPM) of ≥100 in all biological replicates and transcripts with reads covering at least 50% of the coding DNA sequence (CDS). The small RNA (sRNA) fraction was analyzed for the presence of different species of noncoding RNAs (ncRNAs) by aligning the sRNA fraction (reads of <200 nt) with the *H. capsulatum* G186AR strain. These RNA molecules were compared between the strains in order to gain insights into the role of the EV RNA in this fungus and also to determine if there were differences with respect to composition between the two strains with distinct phenotypes.

**Strain-specific content of EV RNA in *H. capsulatum***. We identified a total of 124 mRNA sequences in EV samples from the two strains and carried out paired comparisons between the G186AR and G217B samples. We applied the statistical negative binomial test with filters corresponding to TPM values of ≥100, log2 values of ≥2, and false-discovery-rate (FDR) values of ≤0.05. We observed 93 transcripts enriched in EVs derived from the G217B strain, while 31 transcripts were enriched in the G186AR strain (see Table S1 in the supplemental material). In the G217B-associated transcripts, we observed enrichment in biological processes for vesicle-mediated transport (18%), oxidation-reduction mechanisms (12%), transmembrane transport (11%), and translation (8%) (Fig. 1). In the G186AR strain, the mRNA sequences were enriched only in general cellular and metabolic processes (59%). These results suggest that there are
important differences with respect to the mRNA composition of EVs derived from these two strains of *H. capsulatum*.

**H. capsulatum** EVs contain mRNA fragments and microRNA (miRNA)-like molecules. In addition to the identification of full-length transcripts in EVs, we also detected short reads of averages of 25 to 40 nt in length that aligned consistently in the CDS but at specific positions of the mRNAs (3’ end, 5’ end, or middle sequence); about 50% of these short fragments aligned to the reverse strand, including 172 (G217B) and 80 (G186AR) sequences of this type (Table 1). A total of 172 fragments were represented in the G217B sample compared to only 80 in the G186AR EVs (Table 1). About 47% of the reference mRNA translate proteins of unknown biological processes; this could be explained by the fact that around 33% of the genes annotated in *H. capsulatum* genome code hypothetical proteins and/or do not present a conserved domain, which impedes our current ability to determine specific biological activities. Those associated with DNA metabolism/biogenesis were the second most abundant for both EV samples (22 for G217B versus 16 for G186AR), followed by transport for G217B and by protein modification for both strain EVs. Other processes related to short RNAs identified in both strain EVs were oxidation-reduction, signaling, and carbohydrate and lipid metabolism (Table 1). RNA fragments associated with translation were highly enriched in G217B (n = 11) but not in G186AR (n = 2) EVs, while those related to response to stress were found exclusively in the G217B sample. The corresponding proteins are stress response protein whi2, DNA repair protein rad5, and a thermotolerance protein (Table 1). Analysis of translation-related sequences allowed identification of mRNA fragments associated with distinct steps of the translation process, such as ribosome biogenesis and processing. Other metabolic pathways identified in both strains were protein modification, carbohydrate, and lipid metabolism, signaling, oxidation-reduction, and transmembrane transport, among others (Table 1).
| Feature ID | G217B alignment | G186AR alignment | Sequence description | GO |
|-----------|----------------|-----------------|---------------------|----|
| HCBG_03026 | 5’R | 5’R | Tetrameric peptide-like helical | Amino acid metabolic process |
| HCBG_05660 | MR | CMGC SRPK protein kinase | Amino acid metabolic process |
| HCBG_05782 | MF | Dihydrofolate synthetase fol3 | Cofactor metabolic process |
| HCBG_06582 | MF | Aspartic aminopeptidase | Peptidase activity |
| HCBG_09127 | 3’R / 3’F | Proteasome component C5 | Peptidase activity |
| HCBG_09175 | 5’F | 5’F | Aspartic-type endopeptidase | Peptidase activity |
| HCBG_09182 | MR | Protein kinase | Protein modification process |
| HCBG_09116 | 5’F | Oxidative stress-induced growth inhibitor 2 | Peptidase activity |
| HCBG_00058 | 5’R | Mannosyl-oligosaccharide alpha-mannosidase | Catabolic process |
| HCBG_00633 | 3’R / 3’NS | Class V chitinase | Catabolic process |
| HCBG_03251 | 3’F | Tim-barrel enzyme family protein | Oxidoreductase activity |
| HCBG_04580 | 5’R | Prenyl cysteine carboxyl methyltransferase Ste14 | mRNA processing |
| HCBG_00544 | MF | Ubiquitin conjugating enzyme | Ligase activity |
| HCBG_05116 | 3’R | General stress response protein Whi2 | Response to stress |
| HCBG_01169 | 3’R | DNA repair protein Rad5 | Response to stress |
| HCBG_04793 | 5’R | US small nuclear protein | Chromosome organization |
| HCBG_04436 | 5’F | Flavin-containing monooxygenase | Oxidoreductase activity |
| HCBG_00763 | 3’R / 3’NS | MinD kinetochore complex component Nnf1 | Signal transduction |
| HCBG_03086* | 5’R / F | Ste20 paka protein kinase | Reproduction |
| HCBG_04646* | 3’R | Protein Ras-2 | Signal transduction |
| HCBG_00485 | 3’R | Vacular ABC heavy-metal transporter | Transmembrane transport |
| Feature ID    | G217B alignment | G186AR alignment | Sequence description GO                                      |
|--------------|----------------|-----------------|----------------------------------------------------------|
| HCBG_00680   | 3’F            |                 | Arsenine resistance protein Transmembrane transport       |
| HCBG_00850   | MR             |                 | MFS monooxygenase Transmembrane transport                 |
| HCBG_01089   | 5’F / 5’NS     | 5’R / 5’NS      | Mitochondrial carrier Transport                           |
| HCBG_02374   | 5’R            |                 | Endosomal cargo receptor Vesicle-mediated transport       |
| HCBG_02985   | 5’R            | 5’R             | V-type proton ATPase proteolipid subunit Vesicle-mediated transport |
| HCBG_03067   | 5’R            | 5’R             | Mitochondrial dicarboxylate carrier Transport             |
| HCBG_03738   | MF             |                 | Exocyst complex component Sec10 Vesicle-mediated transport |
| HCBG_04312   | 3’F            |                 | Nonrepetitive nucleoporin Nucleocytoplasmic transport     |
| HCBG_04317   | 5’F            |                 | mRNA transport regulator Transport                         |
| HCBG_04719   | 5’F            |                 | Nucleoporin                                               |
| HCBG_04608   | 3’R            |                 | MFS transporter Transmembrane transport                   |
| HCBG_05671   | MR             |                 | Actin-associated protein Vesicle-mediated transport        |
| HCBG_05941   | 5’F            | 5’R             | Potassium uptake protein Transmembrane transport          |
| HCBG_05942   | MR             |                 | MFS transporter Transmembrane transport                   |
| HCBG_06437   | MF             |                 | Oligopeptide transporter Transport                         |
| HCBG_06658   | MR             |                 | PX domain-containing protein Vesicle-mediated transport    |
| HCBG_07112   | MF             |                 | Ap-2 adaptor complex subunit Vesicle-mediated transport   |
| HCBG_07566   | 3’R            | 3’R / MR        | Actin cytoskeleton-regulatory complex protein Pan1        |
| HCBG_08252*  | 5’F            |                 | MFS multidrug transporter Transmembrane transport          |
| HCBG_09093   | 5’R            |                 | Kinetoplast-associated protein Kap Transmembrane transport |
| HCBG_09150   | 5’R / 3’R      |                 | Cap binding protein Transport                              |
| HCBG_04513   | 5’F            |                 | 3-Oxoacyl-acyl-carrier-protein synthase                    |

DNA metabolism or biogenesis

| Feature ID    | G217B alignment | G186AR alignment | Sequence description GO                                      |
|--------------|----------------|-----------------|----------------------------------------------------------|
| HCBG_00397   | MF             |                 | PHD finger domain Chromosome organization                 |
| HCBG_00799   | 5’F            | 5’F             | C6 zinc finger domain-containing protein Biosynthetic process |
| HCBG_05511   | 3’R            | 3’R             | Transcription factor SteA Reproduction                    |
| HCBG_05417   | MF             |                 | Elongator complex protein 3 Biosynthetic process          |
| HCBG_05986   | 5’F            |                 | G1/S regulator DNA metabolic process                      |
| HCBG_05814   | 3’R            | 3’R             | Histone H2a Chromosome organization                       |
| HCBG_06244   | MF             |                 | Double-strand-break repair protein DNA metabolic process, reproduction |
| HCBG_07395   | MR             |                 | CP2 transcription factor Biosynthetic process             |
| HCBG_07428   | 3’F            |                 | C1f family ribonuclease                                   |
| HCBG_09164   | MF             |                 | C2H2 finger domain transcription factor Biosynthetic process |
| HCBG_09046   | 5’F            |                 | Transcription factor Tau55-like protein DNA metabolic process |
| HCBG_01340   | 3’R            | 3’R             | Formamidopyrimidine-DNA glycosylase Ion binding, lipid binding |
| HCBG_01534   | MF             |                 | Telomere length regulation protein Elg1 DNA metabolic process |
| HCBG_06146   | 5’R / 5’F      | 5’R / 5’F       | Telomerase-binding protein Est1a Ion binding, lipid binding |
| HCBG_07560   | 5’R / 5’F      | 5’R / 5’F       | DNA repair protein protein                                |
| HCBG_05625   | 3’R            | 3’R             | p60-like cell wall                                        |
| HCBG_09024   | MR             |                 | Hlh transcription factor                                  |
| HCBG_06915   | 5’F            | 5’F             | Proline-rich protein-15                                    |

Other/unknown function

| Feature ID    | G217B alignment | G186AR alignment | Sequence description GO                                      |
|--------------|----------------|-----------------|----------------------------------------------------------|
| HCBG_00048   | 5’R            |                 | Hypothetical protein HCBG_00048 Ion binding              |
| HCBG_00453   | 5’R            |                 | MIZ zinc finger protein Ion binding                      |
| HCBG_00947   | 3’F            |                 | Predicted protein                                        |
| HCBG_00975   | 5’R            | 5’R             | ATPase AAA-5 protein Ion binding                         |
| HCBG_01015   | MF             |                 | Predicted protein                                        |
| HCBG_01082   | 3’R / 3’F      | 3’R             | Zinc knuckle domain protein                              |
| HCBG_01086   | 5’R            |                 | Predicted protein                                        |
| HCBG_01127   | 5’R / 3’R      |                 | Predicted protein                                        |
| HCBG_01146   | MF             |                 | Predicted protein                                        |
| HCBG_01161   | MF             |                 | Predicted protein                                        |
| Feature ID | G217B alignment | G186AR alignment | Sequence description | GO |
|----------------|-----------------|-------------------|---------------------|----|
| HCBG_01256  | 3'R             |                    | Conserved hypothetical protein |     |
| HCBG_01258  | MR              |                    | Predicted protein |     |
| HCBG_01500  | MR              |                    | Predicted protein |     |
| HCBG_01656  | MF              |                    | Predicted protein |     |
| HCBG_01888  | 3'R             | 3'R                | Conserved hypothetical protein |     |
| HCBG_01952  | 3'F             |                    | Conserved hypothetical protein |     |
| HCBG_02098  | 5'R             |                    | Protein |     |
| HCBG_02107  | 5'F             |                    | Predicted protein |     |
| HCBG_02158  | 3'F             |                    | Conserved hypothetical protein |     |
| HCBG_02464  | 3'R / 3'F       | 3'F / 3'R / 3'N5   | Carbohydrate-binding module family 48 protein |     |
| HCBG_02569  | MR / MF         | MF                 | Predicted protein |     |
| HCBG_02659  | MR / MF         | MR                 | Predicted protein |     |
| HCBG_02697  | 3'R             | 3'R                | Conserved hypothetical protein |     |
| HCBG_02981  | MF              |                    | Phosphotransferase enzyme family protein |     |
| HCBG_02986  | MF              | 5'F                | Predicted protein |     |
| HCBG_03093  | MR              |                    | PH domain protein |     |
| HCBG_03374  | MF              | MF                 | Glutathione transferase |     |
| HCBG_03658  | 3'R / 3'F       |                    | Conserved hypothetical protein | Helicase activity |
| HCBG_03692  | 3'R / 3'F       |                    | Predicted protein |     |
| HCBG_03693  | MR / MF         | MR / MF            | Predicted protein |     |
| HCBG_03805  | MF              | MF                 | mtDNA inheritance protein |     |
| HCBG_03899  | MR              | MR / 3'R           | WD repeat protein |     |
| HCBG_03911  | 3'R             | 3'R                | Protein |     |
| HCBG_03913  | MR              |                    | Hypothetical protein HCBG_03913 |     |
| HCBG_03980  | MR              |                    | Phosphatidylethanolamine decarboxylase |     |
| HCBG_04009  | MR              |                    | Hypothetical protein HCBG_04009 |     |
| HCBG_04186  | MR              |                    | Conserved hypothetical protein |     |
| HCBG_04193  | 3'R             | 3'R                | Conserved hypothetical protein |     |
| HCBG_04201  | 3'F             | 3'R                | Hypothetical protein HCBG_04201 |     |
| HCBG_04208  | 3'F             | 3'F                | Conserved hypothetical protein |     |
| HCBG_04365  | MF              |                    | Hypothetical protein HCBG_04365 |     |
| HCBG_04371  | 5'R / 5'F       |                    | Bifunctional uridylyltransferase uridylyl-removing enzyme |     |
| HCBG_04380  | 3'R             | 3'R                | Predicted protein |     |
| HCBG_04393  | 3'R             |                    | Protein |     |
| HCBG_04452  | 3'R             | 3'R                | Predicted protein |     |
| HCBG_04780  | 5'R             | 5'R                | Bromodomains-containing protein |     |
| HCBG_04887  | MR              |                    | Predicted protein |     |
| HCBG_05336  | 5'R             |                    | UPF0160 domain protein |     |
| HCBG_05404  | 3'R / 3'F       |                    | Predicted protein |     |
| HCBG_05580  | 3'R             |                    | Methyltransferase domain-containing protein |     |
| HCBG_05638  | 5'R             |                    | Predicted protein |     |
| HCBG_05703  | 5'R             |                    | Conserved hypothetical protein |     |
| HCBG_05744  | 5'F             |                    | T-complex protein 1 subunit beta |     |
| HCBG_05763  | 3'R             | 3'F                | Conserved hypothetical protein |     |
| HCBG_05878  | 3'F             |                    | Hypothetical protein HCBG_05878 |     |
| HCBG_06018  | 5'F             |                    | Cytomegalovirus GH-receptor family |     |
| HCBG_06054  | MR              |                    | Phosphotransferase family protein | Ion binding, kinase activity |
| HCBG_06071  | MF              | MF                 | Protein |     |
| HCBG_06082  | MR              |                    | Conserved hypothetical protein |     |
| HCBG_06114  | 3'F             |                    | Protein |     |
| HCBG_06176  | 3'F             |                    | KH domain protein | RNA binding |
| HCBG_06239  | 5'R             |                    | Nonsense-mediated mRNA decay protein |     |
| HCBG_06270  | MR              |                    | Predicted protein |     |
| HCBG_06364  | MR              |                    | F-box domain-containing protein |     |
| HCBG_06436  | MF              |                    | Predicted protein |     |
| HCBG_06611  | 5'NS            |                    | Predicted protein |     |
| HCBG_06677  | 3'F             |                    | Predicted protein |     |
| HCBG_06927  | 3'R / 3'F       |                    | Predicted protein |     |
| HCBG_07002  | 5'R / 5'F       | 5'R / 5'F          | Ketoreductase |     |
| HCBG_07065  | 5'F             |                    | Predicted protein |     |
| HCBG_07214  | 5'R             | 5'R                | Predicted protein |     |
| HCBG_07247  | MR              |                    | Acryltransferase 3 | Transferring acyl groups |
| HCBG_07296  | MR              | MR                 | Hypothetical protein HCBG_07296 |     |

(Continued on next page)
To gain further insight into the role of EV RNAs, to determine if they could be derived from a miRNA-like pathway, and to assess if they could play a biological role in the recipient cell, we searched for RNA secondary structures, since they are fundamental for gene expression regulation (21). A broad study of RNA structures in distinct cells revealed regulatory effects of the RNA structure throughout mRNA life cycle such as polyadenylation, splicing, translation, and turnover (22, 23). Using the entire range of EV RNA sequencing (RNA-seq) data, a total of 33 RNAs with putative structures were generated by a probability distribution, using a free energy (ΔG) value of less than or equal to \(-7.0\) (Table S2). On the basis of this parameter, we identified transcripts for U3 small nucleolar RNA-associated protein, L-isoaspartate O-methyltransferase, serine/threonine-protein kinase, proteasome component C5, pre-rRNA processing protein Utp22, C-x8-C-x5-C-x3-H zinc finger protein, fungus-specific transcription factor domain-containing protein, and DNA damage-responsive transcriptional repressor RPH1 (Fig. 2; see also Table S2).

**Comparison of EV ncRNA classes in *H. capsulatum* EVs.** We used the ncRNA database from *H. capsulatum* to identify the classes of ncRNA present in EV RNAs. The data analysis revealed 73 different sequences of ncRNA in *H. capsulatum* EVs from the G186AR strain and 38 from the G217B isolate. A total of 33 molecular species were common to both strains, 40 were exclusively identified in the G186AR strain, and the most abundant class of ncRNA found in *H. capsulatum* EVs consisted of tRNAs (Table 2).

**Analysis of proteins putatively associated with RNA metabolism in the EVs.** As a rule, cellular RNAs are covered with proteins and exist as ribonucleoprotein (RNP) complexes. The proteins associated with RNAs are named RNA-binding proteins (RBPs). These proteins participate in several biological processes, ranging from transcription to RNA decay (24). In this context, we investigated the presence of RBPs in the *H. capsulatum* EVs. We analyzed the proteomic EV data available for the G217B strain (25), and we identified 139 proteins related to RNA metabolism (8) (Table 3; see also Table S3). We found many RBPs, such as poly(A) binding protein (PABP), Nrd1, Prp24, and Snd1; splicing factors, exosome complex components, and ribosomal proteins (Table 3; see also Table S3) were identified. In addition, we also found quelling-deficient protein 2 (QDE2), an Argonaute protein important in the RNA machinery in fungi. Because we identified the QDE2 in EVs, we searched for the components of the RNA interference (RNAi) machinery in *H. capsulatum* and compared them with the proteins from *Neurospora crassa* and *Schizosaccharomyces pombe*, which are the fungal species for which the RNAi machinery was best described previously (26, 27). *H. capsulatum* EVs contained one Argonaute protein (QDE2), two Dicer-like proteins, the QIP (quelling interaction protein), and the RNA-dependent RNA polymerase (QDE1) (Table 4).

### TABLE 1 (Continued)

| Feature ID          | G217B alignment | G186AR alignment | Sequence description                  | GO                             |
|---------------------|-----------------|------------------|---------------------------------------|--------------------------------|
| HCBG_07377         | MF              | MR               | Predicted protein                     |                                |
| HCBG_07484         | 3’F             | MR               | Rhomboid family membrane protein      | Peptidase activity             |
| HCBG_07611         | MR / MF         | MR / MF / MNS    | Protein                               |                                |
| HCBG_07676         | 3’R / 3’F       | 3’R / 3’F        | Lyr family protein                    |                                |
| HCBG_07802         | 3’R / 3’F       | 3’R / 3’F        | Predicted protein                     |                                |
| HCBG_07811         | 3’F             | 3’F              | Predicted protein                     |                                |
| HCBG_08059         | MR              | MF               | DUF833 domain protein                 | Protein complex assembly       |
| HCBG_08505         | 3’F             | MF               | Sucrase ferredoxin domain-containing protein |                                |
| HCBG_08661         | MF              | MF               | Predicted protein                     |                                |
| HCBG_08693         | 3’R             | Set domain protein|                                      |                                |
| HCBG_08838         | 5’R             | WW domain        |                                       |                                |
| HCBG_08850         | 5’R             | Integral membrane protein |                       |                                |
| HCBG_09013         | 5’F             | 5’F              | Predicted protein                     |                                |
| HCBG_09099         | 5’R             | 5’R              | Conserved hypothetical protein         |                                |
| HCBG_09144         | MF              | MF               | Predicted protein                     |                                |

*For some transcripts, there was an alignment in specific positions of the mRNA, not covering the entire sequence. 5’, 3’, or M (middle of the mRNA) followed by an “F” or an “R” represents forward (F) or reverse (R) orientation. GO, gene ontology; GPI, glycosylphosphatidylinositol; ID, identifier; mtDNA, mitochondrial DNA.*
Comparisons of cellular RNA versus EV RNA showed a distinct enrichment of molecules in the vesicles. We next assessed the composition of cellular RNA from *H. capsulatum* yeast cells (28) and compared this information to that obtained from analyses of EV-associated RNA composition under the same conditions. There was no correlation between the transcripts with highest expression levels and their presence in the EVs (Table S4). Examples of highly expressed cellular transcripts included histones 4, 2B, and 2A, allergen Aspf4, chaperones, and translation factors, among others (Table S4). In contrast, zinc knuckle domain-containing protein, vacuolar ATP synthase subunit C, Gr15 regulator, thermotolerance protein, histone variant H2A.Z, and proteasome component C5 had an enrichment value of greater than 7,000 in the EVs, while they showed low expression values in the cell (Table S4). The differences in composition between cells and EVs were also evaluated by grouping the transcripts into biological categories.
### TABLE 2 Classes of ncRNA sequences identified in EV preparations from *H. capsulatum* strains G186AR and G217B

| RNA category and ncRNA | G186AR | G217B |
|------------------------|--------|-------|
| rRNA                   |        |       |
| 15S_rRNA               |        | X     |
| NTS1-2                 | X      |       |
| RDN18-1                |        |       |
| RDN18-2                | X      |       |
| RDN25-1                |        |       |
| RDN25-2                | X      |       |
| RDN37-1                |        |       |
| RDN37-2                | X      |       |
| RDN5-1                 | X      | X     |
| RDN5-2                 | X      | X     |
| RDN5-3                 | X      | X     |
| RDN5-4                 | X      | X     |
| RDN5-5                 | X      |       |
| RDN5-6                 | X      | X     |
| RDN58-1                | X      | X     |
| RDN58-2                | X      | X     |
| ncRNA                  |        |       |
| RUF21                  | X      | X     |
| snoRNA                 |        |       |
| snR54                  | X      | X     |
| tRNA                   |        |       |
| tRNA-Ser               |        | X     |
| tRNA-Met               |        | X     |
| tRNA-Gln               |        | X     |
| tRNA-Cys               |        | X     |
| tRNA-Ser               | X      | X     |
| tRNA-Pro               | X      | X     |
| tRNA-Ala               | X      | X     |
| tRNA-Thr               | X      | X     |
| tRNA-Ala               | X      | X     |
| tRNA-Phe               | X      | X     |
| tRNA-Ala               | X      | X     |
| tRNA-Asn               | X      | X     |
| tRNA-Met               | X      | X     |
| tRNA-Arg               | X      |       |
| tRNA-Trp               | X      | X     |
| tRNA-Gly               | X      | X     |
| tRNA-Asp               | X      |       |
| tRNA-Pro               | X      | X     |
| tRNA-Thr               | X      |       |
| tRNA-His               | X      | X     |
| tRNA-Glu               | X      | X     |
| tRNA-Gln               | X      | X     |
| tRNA-Tyr               | X      |       |
| tRNA-Gln               | X      |       |
| tRNA-Gly               | X      |       |
| tRNA-Lys               | X      |       |
| tRNA-Ile               | X      |       |
| tRNA-Leu               | X      |       |
| tRNA-Met               | X      |       |
| tRNA-Gly               | X      |       |
| tRNA-Ile               | X      |       |
| tRNA-Thr               | X      |       |
| tRNA-Lys               | X      |       |
| tRNA-Met               | X      |       |
| tRNA-Val               | X      |       |
| tRNA-Phe               | X      |       |
| tRNA-Ile               | X      |       |
| tRNA-Sec               | X      |       |
| tRNA-Asp               | X      |       |
| tRNA-Thr               | X      |       |

(Continued on next page)
processes (Fig. 3). For the yeast cells, the main pathways were associated with transport, translation, and general metabolic processes (Fig. 3). For the EVs, the enriched pathways were transmembrane transport, protein phosphorylation, and transcription regulation (Fig. 3). This result demonstrates the low levels of correlation between the most highly expressed cellular mRNAs and EV cargo, providing evidence that there might be a mechanism directing the RNA molecules to the EVs.

DISCUSSION

As previously described (17, 18), RNA molecules associated with fungal EVs are remarkably diverse. For instance, mRNAs, tRNA fragments, snoRNAs, small nucleolar RNAs (snRNAs), and miRNA-like molecules were characterized in EVs from *C. albicans*, *C. neoformans*, *P. brasiliensis*, and *S. cerevisiae* (17). We observed similar distributions of RNA molecules in *H. capsulatum* EVs. The comparison between the G186AR and G217B EVs revealed important differences in the variety of mRNAs identified. When the mRNA composition was compared to what was described for other fungi, important similarities were observed. For example, the most abundant biological process identified in G217B EVs was vesicle-mediated transport, which was also the most abundant process in *C. albicans* EVs (17). Molecules required for ribosome biogenesis, which were observed in G217B EVs, belonged to the most highly enriched process in *S. cerevisiae* EVs (17). However, in the comparisons of the ncRNA molecules, different profiles were observed. Most of the ncRNAs in *H. capsulatum* strains derived from tRNAs; a similar profile was obtained with *C. albicans* (17). In addition, almost no snoRNAs were identified in *H. capsulatum*, but this class of ncRNAs was one of the most abundant in the EVs of other fungi (17). Differences in EV composition were observed previously in *C. neoformans*; the EV-associated RNA produced by mutant cells with defective unconventional secretion differed considerably from similar samples produced by wild-type cells (29).

In our study, we identified short reads that aligned specifically to exons; however, these sequences did not correspond to complete mRNAs in the EVs. They instead corresponded to 25-nt-long fragments that were enriched in specific exons of the transcript. These fragments of mRNAs were previously described in human cells (30), where most of the transcripts identified in the EVs corresponded to a fraction of the mRNA with an enrichment of the 3′ UTR of the transcript (30). The results of that human study led to the hypothesis that the mRNA fragments had a role in gene expression regulation in the recipient cells as the secreted mRNA could act as competitors to

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TABLE 2 (Continued)

| RNA category and ncRNA | G186AR | G217B |
|------------------------|--------|-------|
| tRNA-Ile               | X      | —     |
| tRNA-Ser               | X      | —     |
| tRNA-Ser               | X      | —     |
| tRNA-Arg               | X      | —     |
| tRNA-Lys               | X      | —     |
| tRNA-Leu               | X      | —     |
| tRNA-Ser               | X      | —     |
| tRNA-Leu               | X      | —     |
| tRNA-Ala               | X      | —     |
| tRNA-Cys               | X      | —     |
| tRNA-Thr               | X      | —     |
| tRNA-His               | X      | —     |
| tRNA-Tyr               | X      | —     |
| tRNA-Ser               | X      | —     |
| tRNA-Leu               | X      | —     |
| tRNA-Lys               | X      | —     |
| tRNA-Ala               | X      | —     |
| tRNA-Arg               | X      | —     |
| tRNA-Glu               | X      | —     |

*X*, present; —, absent.
| Majority protein ID | Protein name                  | Gene name         |
|---------------------|-------------------------------|-------------------|
| C0NMG7              | QDE2 protein                  | HCBG_03944       |
| C0P170              | Cap binding protein           | HCBG_09150       |
| C0U23               | Exosome complex exonuclease RRP4 | HCBG_03153     |
| C0NMO3              | Exosome complex exonuclease RRP45 | HCBG_04533    |
| C0CT3               | KH domain RNA-binding protein | HCBG_00929       |
| C0UH0               | KH domain RNA-binding protein | HCBG_07001       |
| C0UJ5               | KH domain-containing protein  | HCBG_02352       |
| C0UJS5              | mRNA 3’-end-processing protein RNA14 | HCBG_06689   |
| C0NNW0              | mRNA cleavage and polyadenylation factor CLP1 | CLP1 HCBG_04840 |
| C0NP91              | mRNA decapping enzyme         | HCBG_04971       |
| C0NC87              | mRNA export factor Mex67      | HCBG_00733       |
| C0UJ33              | Nuclear and cytoplasmic polyadenylated RNA-binding protein Pub1 | HCBG_03163 |
| C0QQQ9              | Poly(A)+ RNA export protein   | HCBG_05339       |
| C0SSS5              | Polyadenylate-binding protein (PABP) | HCBG_06205    |
| C0NK4               | Ribonucleoprotein              | HCBG_03744       |
| C0SY4               | RNA binding domain-containing protein | HCBG_06205   |
| C0WH9               | RNA-binding protein            | HCBG_07509       |
| C0N22               | RNA-binding protein            | HCBG_00318       |
| C0P3A1              | RNA-binding protein Nrd1       | HCBG_04981       |
| C0ZI9               | RNA-binding protein Prp24      | HCBG_08569       |
| C0T25               | RNA-binding protein Snd1       | HCBG_06625       |
| C0NMQ0              | RNP domain-containing protein  | HCBG_04027       |
| C0NLQ4              | RRM domain-containing protein  | HCBG_04434       |
| C0UJ27              | Transcription elongation factor Spt6 | HCBG_03157   |
| C0TQ1               | Transcription initiation factor TFIID complex 60-kDa subunit | HCBG_06531 |
| C0RU6               | U1 snRNP-associated protein Usp106 | HCBG_05876    |
| C0Z2                | U1 snRNP-associated protein Usp107 | HCBG_08722    |
| C0BS3               | U2 snRNP auxiliary factor large subunit | HCBG_00569   |
| C0AD4               | U3 small nuclear RNA-associated protein | HCBG_00080   |
| C0ZA3               | U3 small nuclear RNA-associated protein 22 | HCBG_08483 |
| C0NLW4              | U3 snoRNP-associated protein Rrp5 | HCBG_04494   |
| C0P0R0              | U6 snRNA-associated Sm-like protein L5m2 | HCBG_08990   |
| C0P041              | 30S ribosomal protein S10      | HCBG_08883       |
| C0FV8               | 40S ribosomal protein S15      | HCBG_01774       |
| C0X47               | 40S ribosomal protein S18      | HCBG_08039       |
| C0ZD2               | 40S ribosomal protein S20      | HCBG_08512       |
| C0BD0               | 40S ribosomal protein S21      | HCBG_00426       |
| C0UD0               | 40S ribosomal protein S3       | HCBG_06961       |
| C0NL3               | 40S ribosomal protein S4       | HCBG_04423       |
| C0F40               | 40S ribosomal protein S5A      | HCBG_01506       |
| C0NLR5              | 40S ribosomal protein S9       | HCBG_04445       |
| C0TH6               | 5’–3’ exoribonuclease 1 (EC 3.1.13.--) | HCBG_06456   |
| C0K12               | 60S ribosomal protein L1       | HCBG_03662       |
| C0NL2               | 60S ribosomal protein L3       | HCBG_04742       |
| C0CP3               | 60S ribosomal protein L30      | HCBG_00889       |
| C0RD6               | 60S ribosomal protein L5       | HCBG_05566       |
| C0QR6               | 60S ribosomal protein L9b      | HCBG_05346       |
| C0PCC0              | Acyl-RNA-complex subunit       | HCBG_05000       |
| C0KLB               | Alanine-tRNA ligase (EC 6.1.1.7) (alanyl-tRNA synthetase) (AlaRS) | ALA1 HCBG_03698 |
| C0C50               | Alternative oxidase (EC 1.1.1.1) | HCBG_00916     |
| C0D66               | Arginyl-tRNA synthetase        | HCBG_01062       |
| C0T82               | Asparagine-rich protein        | HCBG_06362       |
| C0P94               | Asparaginyl-tRNA synthetase    | HCBG_04974       |
| C0GY7               | Aspartyl-tRNA synthetase       | HCBG_02609       |
| C0NJ3               | ATP-dependent helicase NAM7    | HCBG_04723       |
| C0IT7               | ATP-dependent RNA helicase DOB1 | HCBG_02344    |
| C0AN2               | ATP-dependent RNA helicase EIF4A | HCBG_00178   |
| C0FC7               | Cell cycle control protein     | HCBG_01593       |
| C0T49               | Cleavage and polyadenylation specific factor 5 | HCBG_06329   |
| C0W18               | Clustered mitochondria protein homolog (protein TIF31 homolog) | CLU1 TIF31 HCBG_07348 |
| C0TW5               | Cysteinyl-tRNA synthetase      | HCBG_06595       |
| C0Z4E               | d-Aminoacyl-tRNA deacylase (EC 3.1.1.1) (EC 3.1.1.96) | HCBG_08524   |
| C0SH0               | DNA-directed RNA polymerase II polypeptide | HCBG_06100   |
| C0B61               | DNA-directed RNA polymerase subunit beta (EC 2.7.7.6) | HCBG_00357   |
| C0KS3               | Elicitor protein               | HCBG_03753       |
| C0RY6               | Eukaryotic peptide chain release factor GTP-binding subunit | HCBG_05916   |

(Continued on next page)
| Majority protein ID | Protein name | Gene name |
|--------------------|--------------|-----------|
| C0PO × 7 | Eukaryotic translation initiation factor 3 subunit D (EIF3D) | HCBG_09057 |
| CONE9 | Fibrillarin | HCBG_01425 |
| CONNZ8 | Glutaminyl-tRNA synthetase | HCBG_08668 |
| CONKS5 | Glutamyl-tRNA synthetase | HCBG_03755 |
| CONE28 | Glycyl-tRNA synthetase | HCBG_02121 |
| CONNZ5 | Histidyl-tRNA synthetase | HCBG_04162 |
| CONL64 | Isoleucyl-tRNA synthetase, cytoplasmic | HCBG_03896 |
| CONZPR | Leucyl-tRNA synthetase | HCBG_08644 |
| CONH95 | Leucyl-tRNA synthetase | HCBG_02717 |
| CONI62 | Lysine-tRNA ligase (EC 6.1.1.6) (lysyl-tRNA synthetase) | HCBG_03034 |
| CONM58 | Mitotic control protein dis3 | HCBG_04055 |
| CONBJ8 | mRNA splicing protein PRP8 | HCBG_00494 |
| CONY83 | NAM9+ protein | HCBG_07877 |
| CONG69 | Nucleic acid-binding protein | HCBG_01885 |
| CONUD1 | Phenylalanyl-tRNA synthetase subunit beta | HCBG_06962 |
| CONBD1 | Phenylalanyl-tRNA synthetase subunit beta cytoplasmic | HCBG_00427 |
| CONUP1 | Polymerase II polypeptide D | HCBG_06655 |
| CONNC4 | Pre-mRNA-processing factor 39 | HCBG_04251 |
| CONUB4 | Pre-mRNA-processing protein prp40 | HCBG_03244 |
| CONXM8 | Pre-mRNA-splicing factor | HCBG_08220 |
| CONWL7 | Prolyl-tRNA synthetase | HCBG_04497 |
| CONW72 | Ribonuclease T2-like protein | HCBG_07402 |
| CONEF9 | Ribonuclease Z | HCBG_01127 |
| CONIJ3 | Ribosomal biogenesis protein Gar2 | HCBG_02250 |
| CONHN4 | Ribosomal protein L14 | HCBG_02856 |
| CONI43 | Ribosomal protein L6 | HCBG_03015 |
| CONVX9 | Ribosomal protein S5 | HCBG_07309 |
| CONNB2 | RNA helicase (EC 3.6.4.13) | HCBG_04209 |
| CONEY2 | RNA polymerase II largest subunit | HCBG_01448 |
| CONL28 | RNA polymerase subunit | HCBG_03585 |
| CONYA7 | RNase H domain-containing protein | HCBG_07901 |
| CONH14 | RNP domain-containing protein | HCBG_02636 |
| CONDP9 | RNP domain-containing protein | HCBG_01992 |
| CONCA9 | SAM domain-containing protein | HCBG_00745 |
| CONIEEE | Seryl-tRNA synthetase | HCBG_02184 |
| CONSR2 | Signal recognition particle subunit SRP68 (SRP68) | HCBG_06192 |
| CONDB1 | Small nuclear ribonucleoprotein | HCBG_01107 |
| CONTA0 | Splicing factor 3A subunit 3 | HCBG_06380 |
| CONUB9 | Splicing factor 3B | HCBG_06950 |
| CONBR2 | Splicing factor 3B subunit 1 | HCBG_00558 |
| CONGZ9 | Threonyl-tRNA synthetase | HCBG_02621 |
| CONSB0 | Transfer RNA-Trp synthetase | HCBG_06040 |
| CONL23 | tRNA (cytosine-5-)-methyltransferase NCL1 | HCBG_03853 |
| CONUP2 | tRNA [guanine(37)-N1]-methyltransferase (EC 2.1.1.228) | TRMS HCBG_06656 |
| CONEY10 | tRNA guanylyltransferase | HCBG_01446 |
| CONJ2 | tRNA ligase (EC 6.5.1.3) | HCBG_03322 |
| CONM44 | tRNA pseudouridine synthase | HCBG_04574 |
| CONSG9 | Tyrosine-tRNA ligase (EC 6.1.1.1) (Tyrosyl-tRNA synthetase) | HCBG_06099 |
| CONP46 | Uncharacterized protein | HCBG_04926 |
| CONZF6 | Uncharacterized protein | HCBG_08536 |
| CONIA9 | Uncharacterized protein | HCBG_03081 |
| CONMF3 | Uncharacterized protein | HCBG_04683 |
| CONIP9 | Uncharacterized protein | HCBG_05069 |
| CONK6 | Uncharacterized protein | HCBG_03666 |
| CONAF7 | Uncharacterized protein | HCBG_01563 |
| CONAE1 | Uncharacterized protein | HCBG_01307 |
| CONEC3 | Uncharacterized protein | HCBG_01239 |
| CONJ9 | Uncharacterized protein | HCBG_03369 |
| CONYC3 | Uncharacterized protein | HCBG_07917 |
| CONIB5 | Uncharacterized protein | HCBG_03087 |
| CONYN4 | Uncharacterized protein | HCBG_08264 |
| CONBT4 | Uncharacterized protein | HCBG_00580 |
| CONKE4 | Uncharacterized protein | HCBG_03624 |
| CONGB7 | Uncharacterized protein | HCBG_02389 |
| CONMM1 | Uncharacterized protein | HCBG_04531 |

(Continued on next page)
regulate stability, localization, and translation of mRNAs in target cells (30). In *Mucor circinelloides* cells, the presence of the RNA silencing pathway (sRNA) resulted in the production of both sense and antisense sRNAs (31–33). Sequencing analysis of the sRNA content of this fungus showed the existence of exonic small interfering RNAs (exo-siRNAs) as a new type of sRNA. They were produced from exons of the same genes that are later regulated through the repression of the corresponding mRNA (34). This result agrees with our observation of short reads in the exonic regions of the transcripts. We therefore hypothesize that, similarly to what was described for *M. circinelloides* cells, *H. capsulatum* EV fragments can regulate expression of their own mRNAs.

Of note, we also found a highly represented population of putative exonic RNA in *Paracoccidioides* strains (R. Peres da Silva, L. V. G. Longo, J. P. C. da Cunha, T. J. P. Sobreira, H. Faoro, M. L. Rodrigues, S. Goldenberg, L. R. Alves, and R. Puccia, unpublished data).

As *H. capsulatum* EVs contain different RNA molecules, it is reasonable to hypothesize that proteins that regulate RNA metabolism are also present in the EVs, probably associated with RNA. If validated, this hypothesis could indicate how the RNAs in a specific subset are directed to the vesicles and exported. RNA-binding proteins (RBPs) participate in several biological processes, from RNA transcription to decay (24). We detected a number of RNA-binding proteins in *H. capsulatum* EVs (25). These proteins were also identified in association with EVs in other systems. For example, in the EVs produced by human epithelial cells, 30 RBPs were identified (35), including heterogeneous nuclear ribonucleoproteins (hnRNPs). These proteins are responsible for directing pre-mRNAs in the maturation processes that culminate in transcriptional regulation, alternative splicing, transport, and localization (35). In addition, RBPs in EVs were identified in distinct models as hepatocytes, human embryonic kidney (HEK) cells, and mouse myoblast cells (35–37). Interestingly, one of the RBPs identified in EVs was SND1 (staphylococcal nuclease domain-containing protein 1), which is a main component of the RNA-induced silencing complex (RISC) that plays an important role in miRNA function (37).

Another example of a protein identified in the EVs of *H. capsulatum* and distinct organisms is an endonuclease of the Ago2 family. An infection model with *Plasmodium falciparum* demonstrated that infected red blood cells released EVs containing functional miRNA-Argonaute 2 complexes (38). Moreover, endothelial cells internalized the

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### TABLE 3 (Continued)

| Majority protein ID | Protein name                 | Gene name     |
|---------------------|------------------------------|---------------|
| C0NG47              | Uncharacterized protein      | HCBG_01863    |
| C0NEU7              | Uncharacterized protein      | HCBG_01413    |
| C0NG27              | Valyl-tRNA synthetase        | HCBG_01843    |
| C0P019              | Vip1 protein                 | HCBG_08749    |
| C0NG23              | Ribosome biogenesis protein  | HCBG_01839    |
| C0NGE8              | Ribosome biogenesis protein  | TSR3 HCBG_02420 |
| C0NAE4              | Ribosome biogenesis protein  | YTM1 HCBG_00090 |

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### TABLE 4

Proteins associated with the RNAi machinery in *H. capsulatum* G186AR EVs compared to *S. pombe* and *N. crassa*

| Protein                                      | *H. capsulatum* product | G186AR ID | E value | % identity | % positives |
|----------------------------------------------|-------------------------|-----------|---------|------------|-------------|
| NP_587782.1 (argonaute)                      | QDE2 protein            | HCBG_03944 | 1.00E − 85 | 28         | 45          |
| ESA421221.1 (posttranscriptional silencing protein QDE-2) | QDE2 protein            | HCBG_03944 | 1.00E − 178 | 37         | 53          |
| NP_588215.2 (dicer)                          | Dicer-like protein      | HCBG_01751 | 1.00E − 113 | 28         | 44          |
| EAA434023.3 (dicer-like protein 2 (Neurospora crassa OR74A)) | Dicer-like protein 2    | HCBG_01136 | 3.00E − 97  | 31         | 49          |
| XP_959047.1 (RNA-dependent RNA polymerase (Neurospora crassa OR74A)) | RNA-dependent RNA polymerase | HCBG_06604 | 3.00E − 92  | 31         | 46          |
| XP_964030.3 (RecQ family helicase (Neurospora crassa OR74A)) | Dicer-like protein      | HCBG_01751 | 0.00E + 00  | 45         | 60          |
| ABQ45366.1 (QDE-2-interacting protein (Neurospora crassa)) | QDE-2-interacting protein (QIP) | HCBG_07373 | 2.00E − 50  | 27         | 43          |
*P. falciparum* EVs, and the miRNA-Argonaute 2 complexes were transferred to the cells and acted in regulation of gene expression and in the barrier properties of the recipient cells (38). The Argonaute protein named QDE2 in *H. capsulatum* was identified as enriched in the EVs of the G217B strain. The small silencing RNAs include a variety of molecules, such as microRNAs (miRNAs) and various small interfering RNAs (siRNAs), including exo-siRNAs, endogenous siRNAs (endo-siRNAs), and Piwi-interacting RNAs (piRNAs) (39). Previous studies of small RNAs in fungi identified the RNAi machinery in the fission yeast species *Schizosaccharomyces pombe*, in the budding yeast species *Saccharomyces castellii* and *C. albicans*, and in filamentous fungi (26, 27, 40). One of the best-characterized models is represented by the filamentous fungus *N. crassa* (27, 41–45). The RNAi machinery in that organism functions in defense against transposons (46). A similar process has been described in *C. neoformans*, where RNAi is involved in the regulation of transposon activity and genome integrity during vegetative growth (47). In *N. crassa*, the QDE2 gene encodes an Argonaute protein that is homologous to the rde-1 gene in *C. elegans*, encoding a protein required for double-stranded RNA (dsRNA)-induced silencing (27). The characterization of RNAs associated with QDE2 in *N. crassa* led to the identification of miRNA-like RNAs (miRNAs) in this organism (48). The identification of QDE2 in *H. capsulatum* EVs in association with the small RNAs indicated that the QDE2-miRNA complex might be directed to the EVs and possibly delivered to recipient cells, with the potential to interfere with gene expression regulation and/or cell-cell communication.

Fungal EVs have been implicated in a number of communication processes, including transfer of virulence (49) and antifungal resistance (50). In *Cryptococcus gattii*, pathogen-to-pathogen communication via EVs resulted in reversion of an avirulent phenotype through mechanisms that required vesicular RNA (49). The sequences

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**FIG 3** Gene ontology analysis. The pie charts present the gene ontology of mRNA sequences enriched in *H. capsulatum* cells (A) and in EVs isolated from *H. capsulatum* (B).
required for this process, however, remained unknown. This is an efficient illustration of the potential derived from the characterization of EV-associated RNA in fungi. In this context, our study results provide information from the *H. capsulatum* model that will allow the design of pathogenic experimental models aiming at characterizing the role of extracellular RNAs in fungal pathogenesis.

**MATERIALS AND METHODS**

**Fungal strains and growth conditions.** The *H. capsulatum* strains were subjected to long-term storage at −80°C. Aliquots were inoculated into Ham’s F-12 media (Gibco; catalog no. 21700-075) supplemented with glucose (18.2 g/liter), l-cysteine (8.4 mg/liter), HEPES (6 g/liter), and glutamic acid (1 g/liter) and cultivated at 37°C with constant shaking at 150 rpm. Viability assessments were performed using Janus green 0.02%, and all aliquots used had >99% live yeast cells. EVs were then isolated from fungal culture supernatants as previously described (12).

**sRNA isolation.** Small RNA-enriched fractions were isolated using a mirNeasy minikit (Qiagen) and were then treated with an RNaseasy MinElute cleanup kit (Qiagen), according to the manufacturer’s protocol, to obtain small RNA-enriched fractions. The sRNA profile was assessed in an Agilent 2100 Bioanalyzer (Agilent Technologies).

**RNA sequencing.** Purified sRNA (100 ng) was used for RNA-seq analysis with two independent biological replicates. The RNA-seq analysis was performed using a SOLID 3 Plus platform and an RNA-Seq kit (Life Sciences) according to the manufacturer’s recommendations.

**In silico data analysis.** The sequencing data were analyzed using version 10.1 of CLC Genomics Workbench. The reads were trimmed on the basis of quality, with a threshold Phred score of 25. The reference genomes used for mapping were obtained from the NCBI database (*H. capsulatum* G186AR strain ABB502 and G217B strain ABBT01). The alignment was performed using the following parameters: additional number of bases of upstream and downstream sequences, 100; minimum number of reads, 10; maximum number of mismatches, 2; nonspecific match limit, −2, minimum fraction length, 0.7 for the genome mapping or 0.8 for the RNA mapping. The minimum proportion of read similarity mapped on the reference genome was 80%. Only uniquely mapped reads were considered in the analysis. The libraries were normalized per million, and the expression values for the transcripts were recorded in RPKM (reads per kilobase per million). We also analyzed other expression values, including TPM (transcripts per million) and CPM (counts per million). The statistical test applied was the DGE (differential gene expression) test. For the ncRNA analysis, the database used was the ncRNA database from *Histoplasma capsulatum* (EnsemblFungi G186AR GCA_000150115 assembly ASM15011v1). The secondary structure analysis was performed using the PPFold plugin in CLC Genomics Workbench v. 10.1 and the default parameters. The entire RNA-seq database was subjected to PPFold analysis, and the putative structures were determined. Analysis of the relationship between the profile of RNA sequences detected in this study and the protein composition of *H. capsulatum* EVs was based on results recently obtained with strain G217B using a proteomic approach (25). The cellular RNA used in this analysis was assessed using the Sequence Read Archive (SRA) database (accession numbers SRR2015219 and SRR2015223) (28).

**Data availability.** The data were deposited into the SRA database under study accession number PRJNA514312.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mSphere.00176-19.

**TABLE S1**, XLSX file, 1.4 MB.
**TABLE S2**, XLSX file, 0.01 MB.
**TABLE S3**, XLSX file, 0.1 MB.
**TABLE S4**, XLSX file, 2.1 MB.

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We declare that we have no conflicts of interest.
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