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ORIGINAL ARTICLE

Isobaric tag for relative and absolute quantitation-based comparative proteomic analysis of human pathogenic *Prototheca zopfii* genotype 2 and environmental genotype 1 strains

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Background/Purpose: *Prototheca* species are ubiquitous achlorophyllic microalgae belonging to the family Chlorellaceae, which can cause a wide range of infections in humans and animals. Mainly in individuals with immunologic defects or trauma, *Prototheca* spp. can cause even lethal diseases. However, the exact pathogenic mechanism of *Prototheca* in causing disease remains largely unknown. To investigate the differences between pathogenic and nonpathogenic *Prototheca* spp. genotypes on proteome level, a nonpathogenic *Prototheca zopfii* genotype 1 strain, isolated from cow manure, and a human pathogenic *P. zopfii* genotype 2, isolated from human granulomatous lymphadenitis, were studied.
Methods: Differentially expressed proteins between the two genotypes were quantified by isobaric tag for relative and absolute quantitation-based quantitative proteomics, using liquid chromatography–tandem mass spectrometry.
Results: A total of 245 proteins were identified from the proteomic analysis after data filtering to eliminate low-scoring spectra. Among these, 35 proteins that displayed a significant ($p < 0.05$) 1.5-fold change were considered as differentially expressed proteins.

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Conclusion: The differentially expressed proteins were associated with suppressed energy production and conversion, carbohydrate transport and metabolism, and enhanced translation in the genotype 2 strain, and are thus potentially relevant in the pathogenic mechanism of *P. zopfii* genotype 2, but need further investigation.

Introduction

Prototheca species are ubiquitous, unicellular, achlorophyllic microalgae belonging to the family Chlorellaceae, which can cause a wide range of infections in humans and animals.[1–3] Mainly in individuals with immunologic defects or trauma, *Prototheca* spp. can gain access into the body, and cause even lethal disease.[4–6] The first case of human protothecosis was described by Davies and colleagues in 1964[7] and, since then, over 160 cases have now been reported in literature, with approximately one-third of cases reported in the past 5 years.[5] By contrast, because of their yeast-like appearance when cultured on Sabouraud dextrose agar and their staining patterns, they were considered to be fungal-like organisms.[8] Trends over time demonstrate that reports of protothecosis have become more common as corticosteroid use has become more frequent and the pool of surviving immune-compromised patients has increased.[5] However, the exact role of these organisms as pathogens in human infections remains largely unknown.[2,3]

The taxonomic status of *Prototheca* has evolved over several decades, and six species are currently assigned to the genus *Prototheca*: *Prototheca zopfii*, *Prototheca stagnora*, *Prototheca wickerhamii*, *Prototheca ulmea*, *Prototheca blaschkeae*, and *Prototheca cutis* sp. nov.[9] Among them, *P. zopfii* and *P. wickerhamii* have been associated with human diseases.[10] Based on biochemical, serological, and genetic analyses, *P. zopfii* has been classified into two genotypes, 1 and 2.[3] Interestingly, it is genotype 2 that has been associated with animal and human diseases, while genotype 1 is nonpathogenic.[3,11] The exact mechanism of *P. zopfii* genotype 2 infection remains unknown.[3]

To investigate possible differences between the pathogenic and nonpathogenic *P. zopfii* genotypes on the proteome level, *P. zopfii* genotype 1 strain, isolated from cow manure, and pathogenic *P. zopfii* genotype 2, isolated from human granulomatous lymphadenitis, were chosen for the study. Further detailed information on these two strains can be found respectively in Kano et al[12] and our earlier study.[13]

Methods

Strain cultivation conditions and proteome extraction

Each strain was cultured in Sabouraud dextrose liquid medium at 37°C until the OD_{600} reached 0.6 (mid-logarithmic growth phase), and the experimental design included two biological replicates of each strain. The cells were collected after centrifugation, and washed twice with phosphate-buffered saline. According to the mass ratio of 1:5, the cells were resuspended in 1000 μL lysis solution (8M urea, 0.1% phenylmethylsulfonyl fluoride, 2% CHAPS, 65mM dithiothreitol), and then ultrasound was conducted at 150 W, with 120 periods of 2 seconds and 13 seconds on ice. The lysate was centrifuged at 20800 *g* for 30 minutes at 4°C, and supernatant was collected. An aliquot (3 μL) of supernatant from each lysate was used for protein quantification using the 2-D Quant Kit assay reagents (Amersham, GE Healthcare, Piscataway, NJ, USA) according to the manufacturer's instructions. Finally, a standard curve was generated by plotting the absorbance of the standards against the quantity of protein, and this was used to determine the protein concentration of the samples. The samples were then lyophilized and stored at −80°C.

Protein digestion and isobaric tag for relative and absolute quantitation labeling

The digestion and isobaric tag for relative and absolute quantitation (iTRAQ) labeling of proteins from the four samples (100 μg each) was performed using iTRAQ Reagent 8-plex Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. For digestion, the labeled protein samples from the previous step were redissolved in 2 μL reducing reagent and vortexed at 60°C for 1 hour to break up the disulfide bonds. After this, 1 μL cysteine blocking reagent was added and the contents centrifuged and then spun at room temperature for 30 minutes. An aliquot (35 μL) from each sample was then digested with sequencing grade modified trypsin solution (1:50 w/w added) for 16 hours at 37°C. The aliquots with the digested sample were brought to room temperature after addition of 70 μL of ethanol to each reagent vial. Each of these vials was then appropriately labeled with the iTRAQ tags as follows: two replica clinical isolates of *P. zopfii* (115 and 116 iTRAQ tags) and environmental isolates of *P. zopfii* (119 and 121 iTRAQ tags). Each tube was vortexed and then incubated at room temperature for 1 hour. The labeled samples were combined and dried by vacuum centrifuging.

Two-dimensional nano-liquid chromatography tandem mass spectrometry analysis

After desalting with Sep-Pak Vac C18 cartridge (Waters, Milford, MA, USA), the labeled sample was fractionated by

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