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# Ambient pH Stress Inhibits Spore Germination of *Penicillium expansum* by Impairing Protein Synthesis and Folding: A Proteomic-Based Study

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Spore germination is the first step for fungal pathogens to infect host plants. The pH value, as one of the most important environmental parameters, has critical influence on spore germination. In this study, effects of ambient pH on spore germination were determined by culturing spores of *Penicillium expansum* in medium with pH values at 2.0, 5.0 and 8.0, and involved mechanisms were further investigated through methods of comparative proteomics. The results demonstrated that spore germination of *P. expansum* was obviously inhibited at pH 2.0 and 8.0. Using quadrupole time-of-flight tandem mass spectrometer, 34 proteins with significant changes in abundance were identified. Among them, 17 proteins were related to protein synthesis and folding, and most of them were down-regulated at pH 2.0 and 8.0. Accordingly, lower content of total soluble proteins and higher ratio of aggregated proteins were observed in spores at pH 2.0 and 8.0. In addition, it was found that ambient pH could affect intracellular pH and ATP level of *P. expansum* spores. These findings indicated that ambient pH might affect spore germination of *P. expansum* by changing intracellular pH and regulating protein expression. Further, impairing synthesis and folding of proteins might be one of the main reasons.

**Keywords:** ambient pH • *Penicillium expansum* • spore germination • protein synthesis • protein folding

## Introduction

Fungi have a worldwide distribution and are important for the lives of human beings, both in terms of their ecological and economic roles. In general, there are three distinct phases of the asexual life cycle in fungi: conidial germination, vegetative growth, and conidiation.[1] The asexual spore, or conidium, is critical in the life cycle of many fungi because it is the primary means for dispersion and serves as a 'safe house' for the fungal genome in adverse environmental conditions.[2] When environment is favorable, spore germinates and develops into a new individual.

*Penicillium* is a genus of ascomyceteous fungi. A few species of the genus are important plant pathogens causing decay in various fruits and vegetables.[3,4] Particularly, *Penicillium expansum* can cause diseases in 21 genera of plants,[5] and produce the mycotoxin patulin which has potential carcinogenic effects.[6] Spore germination is the important step for the pathogen to set up infection to host plants. Environmental conditions, such as availability of nutrient, temperature, relative humidity, water activity, and pH, have critical influence on spore germination.[7−11] Manteau et al.[12] reported that pH could determine the survival of living cells, especially on microorganisms since their cells are in direct contact with the environment. Many microorganisms, particularly if they are able to grow over a wide pH range, tailor gene expression to the pH of their growth environment.[13] For example, in *Aspergillus nidulans*, a kind of filamentous fungi, *PacC* encodes a transcription factor which serves as an activator of genes expressed in alkaline conditions and a repressor of those expressed in acidic conditions. Other six proteins have also been reported to involve in the pH-signal transduction and specific gene expression.[14] Spores can show a high germination rate in an optimum pH range, but out of that, the germination capability is markedly impaired. It has been reported that ambient pH inhibits growth of microorganism by affecting enzyme activities, nutrient availability and the proton gradient across the plasma membrane, as well as cell wall remodeling.[15,16] However, most of the investigations concerning the mechanisms by which pH affects germination provided limited information. Therefore, it is important to understand the mechanisms by which environmental pH value inhibits fungal spore germination, in order to set up more effective technologies for controlling diseases.

Proteomic analysis, as a complement to genome and transcriptome analysis, has been proven to be a powerful method for offering a more-direct analysis of cellular response.[17] In our previous study, some crucial proteins related to the pathogenicity of *P. expansum* were successfully characterized using 2-DE-based proteomic approach.[4] Although proteome responses to ambient pH were reported in several microorganisms, such as *Escherichia coli*,[18] *Listeria monocytogenes*,[19]

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*Fusobacterium nucleatum*,[20] *Candida glabrata*,[16] information about the relationship between ambient pH and spore germination is still unavailable. In the present study, a comparative proteomic approach was applied to investigate the effects of ambient pH on spore germination of *P. expansum* and the involved mechanisms. Expressions of proteins involved in fundamental biological processes and other metabolic pathways were differentially regulated under acidic and alkaline pH stresses. Particularly, most of proteins related to protein synthesis and folding were down-regulated. In addition, effects of ambient pH on intracellular pH of spores were also analyzed. These results may provide novel insights into the mechanisms of ambient pH affecting fungal spore germination.

## Materials and Methods

**Organism and Culture Conditions.** The fungal pathogen, *P. expansum* Link was isolated from infected apple fruit showing a typical blue mold symptom. The isolate was purified by a single spore isolation technique and identified based on cultural and morphological characteristics. The pathogen was maintained on potato dextrose agar (PDA) at 4 °C, and inoculated and reisolated from apple fruit before the experiment. The isolate was routinely grown on PDA plates for 14 days at 25 °C. Then spores were collected and suspended in sterile distilled water containing 0.05% (v/v) Tween 20 at the concentration of $1 \times 10^9$ spores $mL^{-1}$ according to the method of Qin et al.[4] PDB medium was buffered with 100 mM $Na_2HPO_4$ and $NaH_2PO_4$ with pH adjusted to 2.0, 5.0, and 8.0 with 1 M NaOH or HCl, respectively. Then aliquots of 1 mL spore suspension were added to 100 mL pH-adapted PDB media in 250-mL conical flasks with the final concentration of $1 \times 10^7$ spores $mL^{-1}$ and cultured at 25 °C on a rotary shaker at 200 rpm for indicated time. After that, the spores were directly sampled for microscopy or collected by centrifugation and used for subsequent experiments, including proteomic analysis.

**Microscopy for Spore Swelling and Germination.** After incubation for 5 and 10 h, spores were sampled from each pH treatment and observed using Zeiss Axioskop 40 microscope (Carl Zeiss, Oberkochen, Germany) to calculate germination rate and determine the spore diameter (5 h) or length of germ tube (10 h). The experiment was conducted three times independently. For each experiment, each treatment was replicated three times by sampling from different cultures. At least 200 spores were randomly selected in each treatment. To facilitate the observation on the shape of germinated spores, fluorescent dye calcofluor white (Sigma, Saint Louis, MO) was used at the concentration of 100 $\mu$g $mL^{-1}$.

**Determination of Intracellular pH.** Intracellular pH of *P. expansum* spores was determined using carboxyfluorescein diacetate succinimidyl ester (CFDASE) according to the method described by Gabier et al.[21] with a slight modification. After they were cultured for indicated time in PDB medium at pH 2.0, 5.0 and 8.0, spores were collected by centrifugation at 3000*g* for 3 min and washed with 100 mM citric phosphate buffer (CPB) with pH equal to the pH of the culture medium. About $2 \times 10^7$ cells were suspended in 1 mL of related CPB buffers, and 10 $\mu$L of CFDASE (Invitrogen, Eugene, OR) at a concentration of 5 mM in dimethyl sulfoxide (DMSO) was added. The suspension was incubated for 30 min at 40 °C. After centrifugation of 3 min at 3000*g*, the pellet was washed twice with CPB buffer, then resuspended in 1 mL of PDB medium, with pH equal to the pH of the sample. The suspension was analyzed with a Hitachi F4500 Fluorescense Spectrophotometer (Hitachi, Tokyo, Japan). The emission wavelength was 520 nm. The fluorescence intensity (FI) was analyzed for excitation wavelengths of 440 and 490 nm. The result was defined as the ratio of the fluorescence intensity for an excitation wavelength of 490 nm to the fluorescence intensity for an excitation wavelength of 440 nm. Fluorescence ratios were converted to intracellular pH using *in vivo* calibration curves. The experiment was conducted three times independently. For each experiment, each treatment was replicated three times by sampling from different cultures. The calibration curve was obtained by loading spores cultured for 5 h in PDB medium at pH 5.0 with CFDASE, as described previously. After loading, stained spores were permeabilized with 70% ethanol for 30 min at 30 °C according to the method of Vindeløv and Arneborg.[22] After centrifugation, the spores were resuspended in PDB of known pH (4.5−8.0) (the pH was adjusted with NaOH or HCl), and the $pH_i$ was allowed to equilibrate with the external pH for 5 min before determination of the fluorescence intensity. The ratios were determined as previously described.

**Determination of ATP Contents.** ATP in spores (about $2 \times 10^8$) was extracted with 50 $\mu$L of 2.5% trichloroacetic acid (TCA) for 3 h at 4 °C. After centrifugation at 10 000*g* for 15 min, 10 $\mu$L of supernatant was diluted with 115 $\mu$L of ATP-free $H_2O$ and 125 $\mu$L of freshly prepared 40 mM Tris-Acetate buffer (pH 8.0, ATP-free). For determination of ATP amounts in the extracts, a luciferin/luciferase kit was used (ENLITEN ATP Assay System, Promega, Madison, WI) according to the protocol of manufacturer. Twenty-five microliters of diluted sample was mixed with 25 $\mu$L of reagent from the kit in tubes. The light emission by the reaction was determined with Veritas Microplate Luminometer (Turner BioSystems, Sunnyvale, CA). The experiment was conducted three times independently. For each experiment, each treatment was replicated three times by sampling from different cultures.

**Preparation of Total Proteins.** After incubation for 5 or 10 h, spores of *P. expansum* were collected by centrifugation (5000*g*, 10 min, 4 °C) and washed 3 times with cold distilled water to remove residual medium. Then, samples were sonificated on ice after adding 1 mL of a extraction buffer containing 0.5 M Tris-HCl, pH 8.3, 2% (v/v) NP-40, 20 mM $MgCl_2$, 2% (v/v) $\beta$-mercaptoethanol, and 1 mM PMSF. The cell debris was removed by centrifugation (25 000*g*, 30 min, 4 °C, 2 times) and the supernatant was extracted with an equal volume of Tris-HCl (pH 7.8) buffered phenol for 30 min. After centrifugation (10 000*g*, 40 min, 4 °C), proteins were precipitated from the final phenol phase with 5 vol of ice-cold saturated ammonium acetate in methanol overnight at −20 °C. The proteins were collected by centrifugation (15 000*g*, 30 min, 4 °C) and washed twice with cold saturated ammonium acetate in methanol and acetone each. The precipitate was air-dried for 1 h at 4 °C and then solubilized in 250 $\mu$L of thiourea/urea lysis buffer containing 2 M thiourea, 7 M urea, 4% (w/v) CHAPS, 1% (w/v) DTT and 2% (v/v) carrier ampholytes of pH 3−10. Protein samples were kept at −80 °C until use. The protein concentration was determined according to Bradford's method using bovine serum albumin as standard.[23]

**Two-Dimensional Gel Electrophoresis.** For two-dimensional (2D) gel electrophoresis, aliquots of 500 $\mu$g of proteins resolved in 250 $\mu$L of sample buffer (7 M urea, 2 M thiourea, 4% [w/v] CHAPS, 1% [w/v] DTT, 2% [v/v] carrier ampholytes [pH 3−10], and 0.001% [w/v] bromphenol blue) were used to rehydrate gel strips (Immobiline DryStrip pH 4−7, 13 cm; GE Healthcare, Piscataway, NJ) for 16 h. The first-dimensional IEF was

performed at 20 °C for a total of 20 kVh on an Ettan IPGphor unit (GE Healthcare) following the manufacturer's instruction. After IEF, the IPG strips were equilibrated in the following two steps and each for 15 min: (a) 150 mM Tris-HCl, pH 6.8, 8 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, and 1% (w/v) DTT; (b) 150 mM Tris-HCl, pH 6.8, 8 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 4% (w/v) iodoacetamide and a trace amount of bromphenol blue. The second-dimensional separation was conducted using 5% stacking gels and 15% polyacrylamide gels at a constant 30 mA/gel. Proteins in the gel were stained with Coomassie Brilliant Blue (CBB) R-250 solution containing 50% (v/v) methanol, 15% (v/v) acetic acid and 0.1% (w/v) CBB R-250.

**Image Analysis of 2D Gels.** Gel images were captured using a flatbed scanner (Amersham Biosciences, Uppsala, Sweden) and saved in TIF format. Comparison of protein expression between samples was performed using Image Master 2D Elite software (Amersham Biosciences). To account for experimental variation, three biological replicate gels resulting from three independent experiments were analyzed for each pH treatment. The amount of a protein spot was calculated based on the volume of that spot which was normalized against total volume of all valid spots. The normalized intensity of spots on three replicate 2D gels was averaged and a two-tailed nonpaired Student's *t*-test was used to determine whether the relative change was statistically significant between samples using SPSS software (SPSS, Inc., Chicago, IL). The differentially expressed protein spots whose expression level was more than two times higher or lower in any of the pH values tested compared to pH 5.0 were manually excised for protein identification.

**In-Gel Digestion, Mass Spectrometry, and Database Searching.** In-gel digestion was performed as our previous reports.[4] Briefly, protein spots excised from the CBB stained gels were destained with 50 mM $NH_4HCO_3$ in 50% (v/v) methanol for 1 h at 40 °C. Then, proteins in gels were reduced with 10 mM DTT in 100 mM $NH_4HCO_3$ and alkylated with 40 mM iodoacetamide in 100 mM $NH_4HCO_3$. After they were washed several times with water and completely dried in a vacuum centrifuge, the gel pieces were digested at 37 °C for 16 h with 5 ng $\mu L^{-1}$ trypsin. Digested peptides were extracted by three changes of 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile. The extracts were pooled and concentrated to 10 $\mu$L in a vacuum centrifuge and desalted with ZipTip$C_{18}$ (Millipore, Bedford, MA). Peptides were eluted into 2 $\mu$L of 0.1% TFA in 50% acetonitrile from the column.

Tandem Electrospray ionization mass spectrometry (ESI-MS/MS) was carried out using a quadrupole time-of-flight mass spectrometer (Q-TOF-2; Micromass, Altrincham, U.K.), equipped with a z-spray source. Prior to sample loading, the instrument was externally calibrated using the fragmentation spectrum of the doubly charged 1571.68 Da (785.84 *m*/*z*) ion of fibrinopeptide B. The peptides were loaded by nanoelectrospray with gold-coated borosilicate glass capillaries (Micromass). The capillary voltage was set to an average of 1000 V, with a sample cone working on 50 V. The collision energy was varied from 14 to 40 V, dependent on the mass and charge state of the peptides. Peptide precursor ions were acquired over the *m*/*z* range 400−1900 Da in TOF-MS mode. Multiply charged (2+ and 3+) ions rising above predefined threshold intensity were automatically selected for MS/MS analysis, and product ion spectra collected from *m*/*z* 50−2000. MS/MS data were processed using ProteinLynx (version 4.0; Micromass) with default parameter to create the peak lists and searched in NCBInr protein databases (version 20090324; 8 097 822 sequences; 2 786 930 639 residues) with Mascot MS/MS Ions Search program on the Matrix Science public Web site (http://www.matrixscience.com). Search parameters were set as taxonomy, fungi; proteolytic enzyme, trypsin; max missed cleavages, 1; fixed modifications, carbamidomethyl (C); variable modifications, oxidation (M); peptide mass tolerance, 1.2 Da; fragment mass tolerance, 0.6 Da. In addition, peptide charge of +2 and +3 and monoisotopic mass was chosen, and the instrument type was set to ESI-QUAD-TOF. Only significant hits as defined by Mascot probability analysis were considered. Mascot uses a probability-based "Mowse score" to evaluate data obtained from tandem mass spectra. Mowse scores were reported as $-10 \times \mathrm{Log}\,10(P)$ where $P$ is the probability that the observed match between the experimental data and the database sequence is a random event. Meanwhile, a Mowse score threshold is also returned by the Mascot program for each individual search, and scores greater than the threshold were considered significant ($p < 0.05$).

**Analysis of Total Soluble Protein and Aggregating Protein.** Spores harvested at an identical cell density were resuspended in 400 $\mu$L of extracting buffer (50 mM potassium phosphate buffer, pH 7.0, 1 mM EDTA, 5% glycerol, 1 mM PMSF) and sonificated on ice. Cell debris was removed by centrifugation at 5000*g* for 30 min. After centrifugation at 15 000*g* for 30 min at 4 °C, soluble proteins in the supernatant were transferred into a new tube for determination of protein content in spores of each treatment. Aggregated protein included in the pellet was isolated and analyzed according to the method of Jang et al.[24] with a slight modification. Briefly, pellets containing the membrane and aggregated proteins were resuspended in 400 $\mu$L of extracting buffer with brief sonification and centrifuged at 15 000*g* for 30 min at 4 °C. The washed pellets were resuspended again in 320 $\mu$L of extracting buffer. Then, 80 $\mu$L of 10% (v/v) NP40 was added to remove membrane proteins, and the aggregrated proteins were isolated by centrifugation (15 000*g*, 30 min, 4 °C). The NP40-insoluble aggregrated proteins extracted from different treatments were washed again with extracting buffer, and finally resuspended with the same volume of the lysis buffer (as described in Preparation of Total Proteins) for gel analysis. To investigate the ratio of aggregated protein to total soluble protein, loading amount of aggregated protein in different treatments were calculated according to the ratio of the extracted amount of soluble protein content (pH 2/pH 5/pH 8). The analysis of soluble protein content and aggregated protein was performed with three biological replicates.

## Results

**Swelling and Germination of *P. expansum* Spores under Different pH.** In preliminary experiment, we found that pH value of unbuffered medium declined with spore germination of *P. expansum*, especially under alkaline conditions. When spores were cultured for 10 h, the pH of PDB medium declined from 8.0 (adjusted by NaOH) to 7.5. It was reported that *P. expansum* could acidify environment or host tissue by secretion of organic acid, such as citric acid, fumaric acid, and oxalic acid.[25] To resist the growth-induced pH shift, phosphate buffered PDB media at different pH values were used in this study as described in Materials and Methods.

In the phosphate buffered PDB system, pH 5.0 was optimum for spore germination, in which *P. expansum* spores began to germinate after 6 h and germination rate reached about 90%

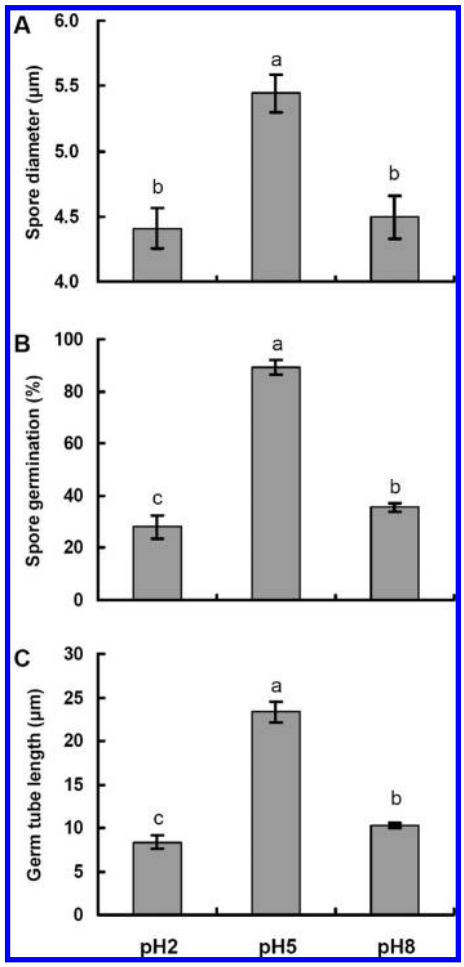


**Figure 1.** The effects of ambient pH on the swelling and germination of *P. expansum* spores. (A) Spore diameter of *P. expansum* after 5 h of culture in PDB media with different pH. (B) Germination rate and (C) germ tube length of *P. expansum* after 10 h of culture in PDB media with different pH. The data represent mean ± standard deviation from three different experiments. Columns with different letters are significantly different from each other by the least significant difference test ($p < 0.05$).

after 10 h. To investigate the effects of ambient pH on spore germination, the other two pH values, that is, pH 2.0 and 8.0, representing acidic and alkaline pH stresses were chosen, respectively, according to our preliminary experiments (data not shown). In the following content, the treatments of pH 2.0, 5.0, and 8.0 are abbreviated as pH 2, 5, and 8, respectively. As shown in Figure 1, compared with pH 5, the swelling (Figure 1A), germination (Figure 1B), and germ tube elongation (Figure 1C) of *P. expansum* spores were significantly inhibited at pH 2 and 8, indicating the spore germination was markedly delayed at pH 2 and 8. Moreover, some differences in morphology of germinated spore were observed among different pH treatments. After 10 h of culture, almost all spores in pH 5 formed an obvious large central vacuole, whereas a few small vacuoles were observed in spores at pH 2 and 8 (Figure 2A–C). Compared with spores in pH 5 (Figure 2D), two or three germ tubes emerged from spores under pH 2 condition, partial spores extended germ tubes when they did not swell enough (Figure 2E–G). The morphology of germ tube in pH 8 was similar to that in pH 5, but length of germ tube was distinctly shorter (Figure 2H,I).

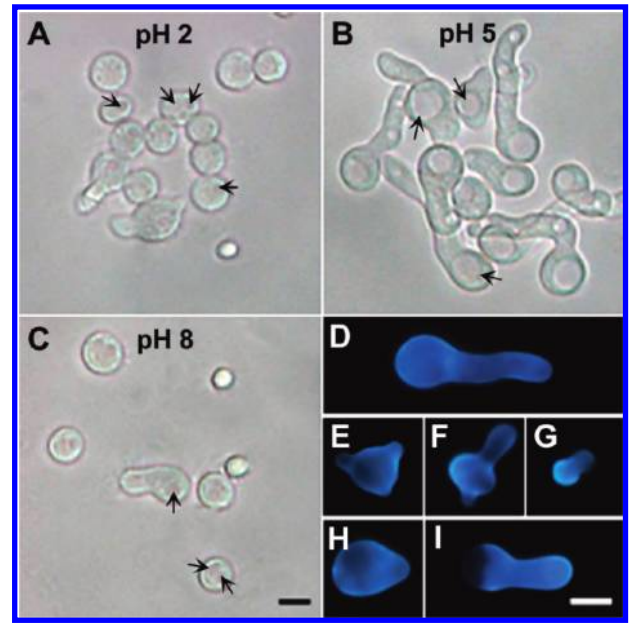


**Figure 2.** Microscopic observation of *P. expansum* spores after 10 h of culture in PDB media with different pH. (A–C) Morphology of spores in indicated pH treatments observed in light field. Arrows indicate the vacuoles of spores. (D–I) Morphology of germinated spores in different pH treatments observed in fluorescent field after being stained with calcofluor white as described in Materials and Methods. (D) Morphology of germinated spores at pH 5. (E–G) Morphology of germinated spores at pH 2. (H and I) Morphology of germinated spores at pH 8. Bar = 10 $\mu$m.

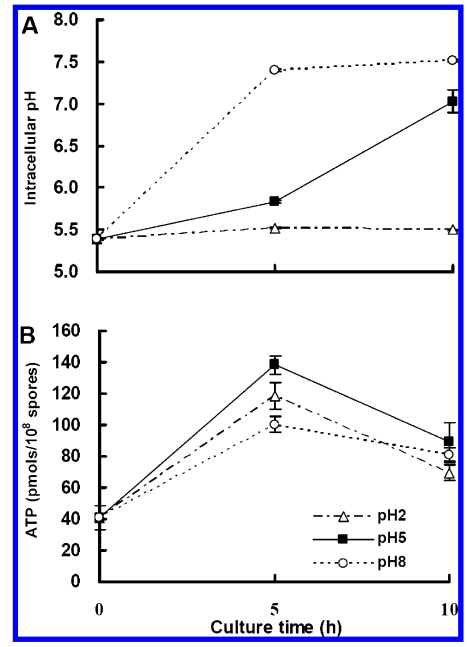


**Figure 3.** Changes of intracellular pH (A) and ATP content (B) in *P. expansum* spores under different pH conditions during germination. The data represent mean ± standard deviation from three different experiments.

**Intracellular pH and ATP Levels of *P. expansum* Spores.** Ambient pH showed significant effect on intracellular pH ($pH_i$) of *P. expansum* spores (Figure 3A). During the germination, $pH_i$ of *P. expansum* spores increased from 5.4 to 7.0 under pH 5. At pH 8, $pH_i$ of spores quickly increased to 7.4 after 5 h of

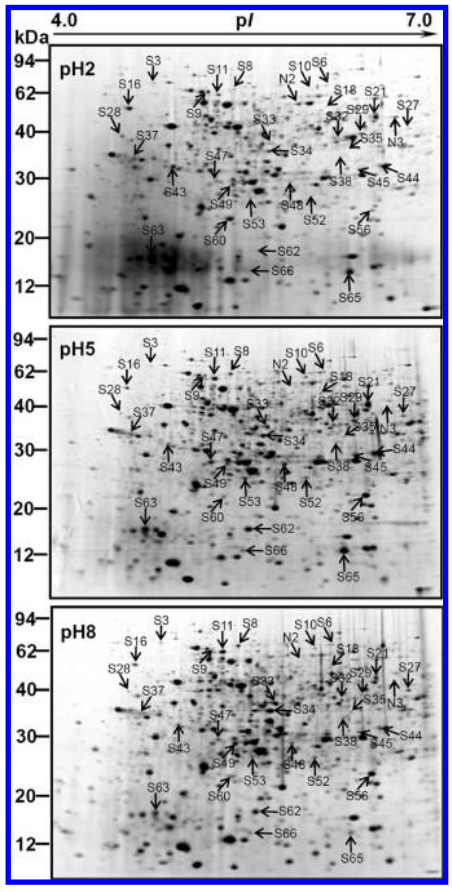


**Figure 4.** Two-dimensional patterns of proteins from *P. expansum* spores after 5 h of culture under different pH conditions. Protein extraction and 2D electrophoresis protocol were described in Materials and Methods. Arrows indicate proteins identified by MS/MS, which changed in abundance more than 2-fold between treatments of pH 5 and any other pH. The protein spots are numbered, corresponding to those in Table 1.

culture, whereas that of spores at pH 2 was lower than 5.5 after 10 h of culture.

At first, ATP levels of *P. expansum* spores presented an increasing trend and subsequently decreased among all treatments (Figure 3B). The ATP level in spores at pH 5 was higher than that in spores at pH 2 and 8.

**Proteome Analysis.** Proteins extracted from *P. expansum* spores cultured in media at pH 2, 5, and 8 were separated using 2D gel electrophoresis. More than 700 protein spots were detected in each gel after ignoring very faint spots and spots with undefined shapes and areas using Image Master 2D Elite software (Figure 4). In the present study, we paid more attention to the differential spots at 5 h (the time point before germination) because they are considered to be more important for spore germination. Comparative analysis of proteome was performed with expression profile at pH 5 as control, and protein spots shown statistically significant ($p < 0.05$) changes of more than 2-fold in relative abundance were selected as differential spots. A total of 75 proteins were differentially expressed in spores under pH 2 and 8 after 5 h of culture. Of them, 58 spots with relatively higher abundance were analyzed through MS/MS and NCBInr database searching and 34 spots were identified with Mowse scores significantly higher than the threshold ($p < 0.05$). The source organism of almost all the identified proteins was *Penicillium chrysogenum* Wisconsin 54-1255 (Table 1) whose genome sequence information was released recently.[26] The MS/MS peptide sequence of the identified proteins is summarized in Supplemental Table 1. Of the 34 identified proteins, 7 proteins were up-regulated and 18 proteins were down-regulated at pH 2, while 4 proteins were up-regulated and 7 proteins were down-regulated at pH 8. Among them, 2 proteins (S3 and 63) were differentially expressed at both pH 2 and 8. In addition, two pairs of spot (S47 and 53, S11 and 66) were identified as the same protein, respectively. Of them, location of S47 and 53 in the gels differed slightly in molecular mass and p*I* (Figure 4), which might be due to a protein truncation event. The experimental molecular mass of S66 was much smaller than that of S11 and the theoretical value of the protein, suggesting S66 might be a fragment of the protein. These identified spots were categorized into 5 groups, including protein synthesis (8 spots), protein folding (9 spots), DNA replication, transcription and protein transport (3 spots), metabolic enzymes (11 spots), and proteins with unknown function (3 spots) (Table 1).

First, it is notable that the abundance of 8 proteins related to protein synthesis changed in pH 2 and 8. Of the 8 proteins, 4 proteins (S43, 60S acidic ribosomal protein P0; S47 and 53, 40S ribosomal protein S0; S63, 40S ribosomal protein S12) were components of ribosomes; 2 proteins (S6, Glycyl-tRNA synthetase 1; S10, aspartyl-tRNA synthetase Dps1) were aminoacyl tRNA synthetase. Another 2 proteins, Elongation factor 2; (S32) and Guanine nucleotide-binding protein beta subunit-like protein (S44), were related to peptide chain elongation and translation efficacy.

Another large group consists of 9 proteins involved in protein folding and most of them belong to molecular chaperones HSP 70 family and folding catalysts, including heat shock protein BiP (S3), heat shock protein STI1 (S8), mitochondrial heat shock protein SSC1 (S9), heat shock protein SSB2 (S11, 66), heat shock protein 70 (S38), protein disulfide-isomerase (PDI, S16), peptidyl-prolyl *cis*−*trans* isomerase D (PPIase, S27), and nascent polypeptide-associated complex subunit beta-1 (NAC-beta-1, S62).

It is interesting that 17 identified spots (accounting for 50% of total identified spots) in the present study were related to protein synthesis and folding. Changes in abundance of those proteins were shown in Figure 5 in a time dependent manner. All proteins related to protein synthesis were down-regulated under both pH 2 and 8 at 5 h except for 60S acidic ribosomal protein P0 (S43, up-regulated at pH 2) (Figure 5A). Among them, the relative abundances of two aminoacyl tRNA synthetases (S6, 10) at pH 2 were over 2-fold lower than those of proteins at pH 5 at both 5 and 10 h. Of the proteins related to protein folding, two spots (S3 and 16) were up-regulated under both pH 2 and 8; others were down-regulated under pH 2 and/or 8 at 5 h. After 10 h of culture, expressions of 5 spots (S8, 9, 11, 27, and 62) were significantly repressed at both pH 2 and 8. Among these spots, S62 was down-regulated under both pH 2 and 8 during spore germination; moreover, it was absent in the gels of pH 2 treatment at 5 h.

Proteins related to DNA replication, transcription and protein transport also revealed differential abundances (the third group, Table 1). Among them, spots S37 and 52 were proteins related to DNA replication, repair and transcription. S56 is a multifunctional protein involved in intracellular protein transport, nucleocytoplasmic transport, and regulation of transcription.

**Table 1.** Proteins Identified in *P. expansum* Spore by Quadrupole Time-of-Flight Tandem Mass Spectrometry

| spot | protein name | NCBI accession | theo. $M_r$ (kDa)/p$I^a$ | expt. $M_r$ (kDa)/p$I^b$ | species | Mascot score[c]/ threshold | NP[d] | SC[e] (%) | pH 2 vs pH 5[f] | pH 8 vs pH 5[g] |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | **Protein Synthesis** | | | | | | |
| S43 | 60S acidic ribosomal protein P0 | gi\|211585945 | 33.1/4.90 | 35.1/4.9 | *P. chrysogenum* | 216/47 | 6 | 25 | 2.39 | 0.74 |
| S6 | Glycyl-tRNA synthetase 1 | gi\|211587397 | 147.3/5.88 | 65.6/6.1 | *P. chrysogenum* | 85/46 | 3 | 2 | 0.43 | 0.79 |
| S10 | Aspartyl-tRNA synthetase | gi\|211583071 | 62.2/5.76 | 62.4/6.0 | *P. chrysogenum* | 182/47 | 6 | 17 | 0.31 | 0.60 |
| S32 | Elongation factor 2 | gi\|211582639 | 94.5/6.30 | 41.7/6.2 | *P. chrysogenum* | 116/47 | 3 | 3 | 0.45 | 0.72 |
| S44 | Guanine nucleotide-binding protein beta subunit-like protein | gi\|211591374 | 35.3/6.31 | 32.6/6.5 | *P. chrysogenum* | 277/48 | 7 | 23 | 0.52 | 0.41 |
| S47 | 40S ribosomal protein S0 | gi\|211584478 | 32.5/4.79 | 30.0/5.2 | *P. chrysogenum* | 357/47 | 9 | 43 | 0.48 | 0.62 |
| S53 | 40S ribosomal protein S0 | gi\|211584478 | 32.5/4.79 | 26.0/5.5 | *P. chrysogenum* | 155/47 | 6 | 30 | 0.46 | 0.69 |
| S63 | 40S ribosomal protein S12 | gi\|211586810 | 16.5/4.86 | 16.5/4.7 | *P. chrysogenum* | 183/47 | 4 | 40 | 0.38 | 0.50 |
| | | | | **Protein Folding** | | | | | | |
| S3 | Heat shock protein BiP | gi\|211582507 | 73.7/4.81 | 68.9/4.8 | *P. chrysogenum* | 90/46 | 3 | 5 | 2.01 | 2.02 |
| S16 | Protein disulfide-isomerase | gi\|211589871 | 56.6/4.68 | 56.9/4.6 | *P. chrysogenum* | 240/47 | 10 | 28 | 2.50 | 1.77 |
| S8 | Heat shock protein STI1 | gi\|211590015 | 62.6/5.33 | 64.1/5.4 | *P. chrysogenum* | 302/46 | 8 | 21 | 0.26 | 1.03 |
| S9 | Heat shock protein SSC1, mitochondrial | gi\|211592918 | 72.6/5.58 | 63.2/5.2 | *P. chrysogenum* | 439/48 | 9 | 18 | 1.58 | 0.47 |
| S11 | Heat shock protein SSB2 | gi\|211592047 | 67.0/5.32 | 62.0/5.3 | *P. chrysogenum* | 977/46 | 20 | 52 | 0.40 | 0.89 |
| S27 | Peptidyl-prolyl *cis*−*trans* isomerase D | gi\|211587757 | 41.3/5.91 | 44.5/6.7 | *P. chrysogenum* | 124/46 | 4 | 18 | 0.46 | 1.13 |
| S38 | Heat shock protein 70 | gi\|211592088 | 69.69/5.03 | 35.2/6.2 | *P. chrysogenum* | 384/47 | 10 | 21 | 0.44 | 1.11 |
| S62 | Nascent polypeptide-associated complex subunit beta | gi\|211593238 | 16.9/5.80 | 16.7/5.5 | *P. chrysogenum* | 135/47 | 3 | 25 | Lost[h] | 0.61 |
| S66 | Heat shock protein SSB2 | gi\|211592047 | 67.0/5.32 | 13.7/5.5 | *P. chrysogenum* | 133/46 | 4 | 7 | Lost | 0.82 |
| | | | | **DNA Replication, Transcription and Protein Transport** | | | | | | |
| S37 | Proliferating cell nuclear antigen (PCNA) | gi\|211585905 | 33.5/4.93 | 38.0/4.6 | *P. chrysogenum* | 261/47 | 6 | 30 | 0.60 | 0.49 |
| S52 | Transcription initiation factor TFIID subunit 14 | gi\|211589487 | 26.4/5.73 | 26.0/6.0 | *P. chrysogenum* | 66/48 | 1 | 8 | 0.49 | 1.00 |
| S56 | GTP-binding nuclear protein GSP1/Ran | gi\|211591187 | 24.4/6.45 | 22.4/6.5 | *P. chrysogenum* | 251/48 | 5 | 29 | 0.18 | 0.84 |
| | | | | **Metabolic Enzymes** | | | | | | |
| N2 | Glucose-methanol-choline oxidoreductase | gi\|211585664 | 66.0/5.76 | 56.9/5.9 | *P. chrysogenum* | 363/47 | 7 | 16 | New[i] | —[j] |
| S34 | Ketol-acid reductoisomerase, mitochondrial | gi\|211584596 | 44.2/8.79 | 37.8/5.6 | *P. chrysogenum* | 475/48 | 9 | 25 | 0.70 | 2.14 |
| S49 | Malate dehydrogenase precursor | gi\|211581963 | 34.3/5.44 | 29.3/5.4 | *P. chrysogenum* | 104/47 | 2 | 7 | 1.49 | 2.12 |
| S60 | Fatty acid regulation protein like protein An14g07060 | gi\|211589332 | 24.3/5.33 | 22.2/5.3 | *P. chrysogenum* | 73/47 | 3 | 18 | 2.85 | 1.62 |
| S18 | Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial | gi\|211582337 | 62.6/6.84 | 54.2/6.1 | *P. chrysogenum* | 94/47 | 2 | 5 | 0.40 | 0.92 |
| S21 | NADP-specific glutamate dehydrogenase | gi\|41017058 | 50.0/6.00 | 49.4/6.5 | *P. chrysogenum* | 539/47 | 11 | 36 | 0.74 | 0.34 |
| S28 | Endochitinase 1 precursor | gi\|211583995 | 44.1/4.49 | 45.7/4.5 | *P. chrysogenum* | 222/47 | 4 | 13 | 2.74 | 1.29 |
| S29 | NADP-dependent alcohol dehydrogenase C | gi\|211587686 | 39.3/7.06 | 42.8/6.3 | *P. chrysogenum* | 217/48 | 4 | 12 | 0.81 | 0.30 |
| S35 | Tyr-inhibited DAHP synthase | gi\|211589228 | 40.2/6.05 | 36.7/6.3 | *P. chrysogenum* | 78/48 | 3 | 10 | 0.44 | 1.17 |
| S45 | NAD dependent epimerase/dehydratase family protein | gi\|211590221 | 35.2/6.06 | 32.1/6.3 | *P. chrysogenum* | 59/47 | 2 | 6 | 1.26 | 2.05 |
| S48 | Adenylylsulfate kinase AAA81521 | gi\|211583872 | 23.6/6.02 | 30.0/5.8 | *P. chrysogenum* | 111/47 | 5 | 30 | 0.27 | 0.58 |
| | | | | **Unknown Function** | | | | | | |
| N3 | Catalase, putative | gi\|121704421 | 55.5/6.99 | 48.7/6.6 | *Aspergillus clavatus* | 226/46 | 6 | 15 | New | — |
| S33 | Hypothetical protein YGR086c | gi\|211587175 | 38.8/5.49 | 40.5/5.7 | *P. chrysogenum* | 417/47 | 8 | 34 | 0.31 | 1.07 |
| S65 | Hypothetical protein CipC | gi\|211590389 | 12.5/5.77 | 14.0/6.3 | *P. chrysogenum* | 64/47 | 2 | 31 | 0.91 | 0.29 |

[a] Theo. $M_r$ (kDa)/p*I*, theoretical molecular mass and isoelectric point based on amino acid sequence of the identified protein. [b] Expt. *Mr* (kDa)/p*I*, experimental molecular mass and isoelectric point estimated from the 2D gels. [c] Mascot scores greater than the threshold are statistically significant ($p < 0.05$). [d] NP, the number of matched peptides. [e] SC, amino acid sequence coverage for the identified proteins. [f] pH 2 vs pH 5, average fold change of relative abundance of specific spot at pH 2 versus pH 5 from three biological repeats. [g] pH 8 vs pH 5, average fold change of relative abundance of specific spot at pH 8 versus pH 5 from three biological repeats. [h] Lost, corresponding spot appeared in the 2D gels at pH 5, but not at pH 2. [i] New, corresponding spot appeared in the 2D gels at pH 2, but not at pH 5. [j] —, corresponding spot disappeared in the 2D gels at both pH 5 and 8.

In addition, other 14 spots were identified as metabolic enzymes and hypothetical proteins (the last two groups, Table 1).

**Changes in Soluble Protein Content and Protein Aggregation of *P. expansum* Spores.** To further investigate whether protein synthesis was impaired by the down-regulation of involved proteins, we determined the soluble protein content of *P. expansum* spores. The soluble protein content in spore lysates was observed to decrease at both pH 2 and 8, compared to pH 5 (2- and 4-fold at 5 and 10 h, respectively) (Table 2). This was indicative of more severe pH stress-related impairment of protein synthesis at highly acidic and basic pH values.

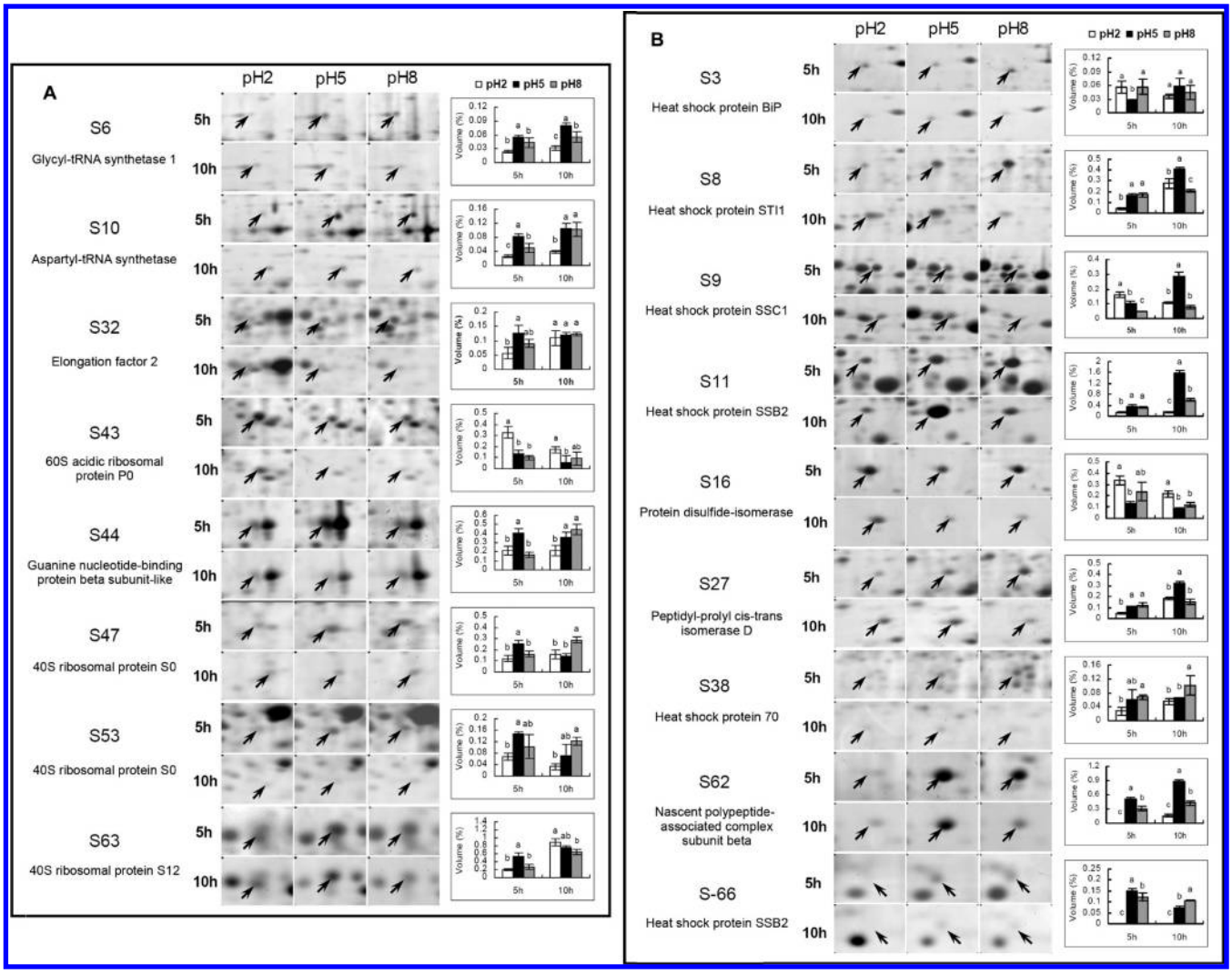


**Figure 5.** Abundance variance of protein synthesis (A) and protein folding (B) related proteins in *P. expansum* spores under different pH conditions after 5 and 10 h of culture. Protein extraction and 2D electrophoresis protocol were described in Materials and Methods. The number of each protein spot corresponds to its listing in Table 1. The spot volume was normalized as a percentage of the total volume of all spots on the corresponding gel. The graph represents an average of three biological replicates. Bars represent standard deviations of the mean. Columns with different letters are significantly different from each other by the least significant difference test ($p < 0.05$).

**Table 2.** Changes in Content of Total Soluble Protein in *P. expansum* Spores under Different pH Conditions

| | protein content ($\mu$g/10$^8$ spores) | | |
|---|---|---|---|
| treatments | 0 h | 5 h | 10 h |
| pH2 | 10.17 ± 1.55a[a] | 40.14 ± 1.70c | 78.44 ± 3.86b |
| pH5 | 10.17 ± 1.55a | 78.77 ± 3.46a | 302.87 ± 6.05a |
| pH8 | 10.17 ± 1.55a | 48.45 ± 2.40b | 89.12 ± 4.24b |

[a] The data represent mean ± standard deviation from three different experiments. Values of each column with different letters are significantly different from each other by the least significant difference test ($p < 0.05$).

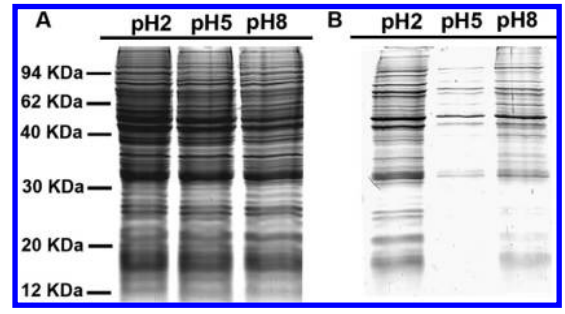


**Figure 6.** Protein aggregation of *P. expansum* spores under different pH conditions. Total soluble protein and aggregated protein were extracted as described in Materials and Methods from *P. expansum* spores cultured for 10 h at pH 2, 5 and 8, and analyzed by 12% SDS-PAGE. (A) Gel image of total soluble protein and (B) aggregated protein under different pH conditions staining with Coomassie Brilliant Blue R-250, respectively.

Several proteins involved in the protein folding were identified as described above. The differential expressions of these proteins at pH 2 and 8 might affect the formation of correct conformation of the proteins. The usual fate of misfolded proteins is to form aggregates.[27] In the study, the aggregated proteins were extracted from *P. expansum* spores after 10 h of culture, and analyzed by SDS-PAGE to investigate the effects of pH stress on protein folding during the germination. As shown in Figure 6, only a few aggregated protein bands were observed in spores at pH 5. These protein bands were also detected in spores at pH 2 and 8 with enhanced level of aggregation. Further, a number of other aggregated protein

bands were induced at pH 2 and 8. These results suggest that the ratio of aggregated protein to soluble protein increased under pH stresses. Moreover, the level of aggregated protein under pH 2 was higher than that of pH 8. Three protein bands with molecular mass ranging from 20 to 30 kDa were specifically induced at pH 2.

## Discussion

In this study, we found that the spore germination of *P. expansum* was markedly decreased under pH 2 and 8 conditions (Figure 1). Moreover, the tube elongation and morphology of germinated spore were also affected under these pH stresses (Figures 1 and 2). To investigate the involved mechanism by which spore germination was inhibited, we analyzed the effects of ambient pH on intracellular pH ($pH_i$) with pH-dependent fluorescent probe and employed high resolution 2D gel electrophoresis to characterize the response in proteome during spore germination. We found that ambient pH has an important effect on the $pH_i$ of *P. expansum* spores (Figure 3A). By affecting activity of metabolic enzymes and synthesis of DNA, RNA, and proteins, $pH_i$ shows an important role in the maintenance of normal cell function.[28] Normally, $pH_i$ is tightly controlled within a narrow range largely by ion transporters and a high buffering capacity of cytosol.[28,29] The changes in $pH_i$ might not only affect the spore germination of *P. expansum* by interfering various biological processes as described above, but also directly affect the energy level in spores (Figure 3B). To maintain the pH homeostasis in cells, $H^+$-ATPases, as the major regulator, need to be activated.[30] The increased activity of $H^+$-ATPases could reduce the intracellular ATP pool because the process of proton transport is ATP dependent, which might contribute to inhibiting spore germination due to the limited amount of ATP available for other cellular activities. Interestingly, we found that $pH_i$ of *P. expansum* increased from 5.4 to 7.0 during spore germination under the optimum pH condition (pH 5) (Figure 3A), which is consistent with the previous result reported by Setlow and Setlow.[31] They observed that the pH within dormant spores of *Bacillus cereus* and *Bacillus megaterium* rose from 6.3−6.4 to 7.3−7.5 upon germination, and considered that the elevation of $pH_i$ was related to the activation of metabolism.

Through ESI-MS/MS, 34 differentially expressed proteins were identified in the present study (Table 1). Among them, we identified 8 crucial proteins related to different aspects of protein synthesis, and 7 of them were down-regulated at pH 2 or 8. Of the 8 proteins, 3 spots representing 2 components of small ribosomal subunit (40S ribosomal protein S0 and S12) were down-regulated, and one acidic ribosomal protein P0 of the large subunit was up-regulated. It is well-known that ribosomes are the machines of protein biosynthesis and consist of a small (40S) and large (60S) subunit in eukaryotes. The decrease in expression of small subunit components and increase in large subunit component may reflect the imbalance in 40S/60S subunit ratio, resulting in the decrease of rate of protein synthesis.[32] Of the remaining down-regulated proteins, Glycyl- and Aspartyl-tRNA synthetases belong to the family of aminoacyl tRNA synthetase, which are essential enzymes responsible for accurately attaching the correct amino acid onto the cognate tRNA molecule in the first step of protein synthesis and providing the elongating polypeptide chain with amino acids in the form of aminoacyl-tRNAs.[33] Elongation factor 2 is responsible for catalyzing the translocation of ribosome along with mRNA.[34] Guanine nucleotide-binding protein beta subunit-like protein, which locates at 40S ribosomal subunit, may directly influence the efficiency and specificity of translation.[35,36] These results suggested that ambient pH might impair the *de novo* synthesis of protein by affecting the various steps in the process, such as the ribosome biogenesis, aminoacyl tRNA synthesis, and polypeptide chain elongation. Protein synthesis has been proven to be required for spore germination in *Neurospora crassa*, *A. nidulans* and *Fusarium solani* using either protein-synthesis inhibitors or temperature-sensitive mutants.[2,37,38] In this study, germination of *P. expansum* spores was also inhibited by cycloheximide, a protein synthesis inhibitor of eukaryotes (Supplemental Figure 1), indicating that spore germination of the fungus was also protein synthesis-dependent. These results indicate that impairing protein synthesis may be one of the main reasons that spore germination of *P. expansum* is inhibited under pH stress. The results of determination of soluble protein contents (Table 2) showed that protein contents actually decreased under both acidic and alkaline pH stresses, which supported our speculation about the impairment of protein synthesis. Moreover, the difference in protein content between spores at pH 5 and those at pH 2 or 8 gradually increased with the culture time, which might be caused by the delay of spore germination under pH stress.

To actively perform their normal functions, *de novo* synthesized polypeptide chains need to be correctly folded.[39,40] Many nascent polypeptides only reach partially folded structures during biosynthesis and are aggregation-prone, due to the exposure of hydrophobic residues.[39] Molecular chaperones are required to protect nonnative protein chains from misfolding and aggregation, and form their unique 3D conformation. In the study, we identified 6 proteins (STI1, SSB2, BiP, PDI, PPIase, and NAC-beta-1) related to nascent polypeptide stability and folding. Among them, NAC-beta-1 is one of the two components of the nascent polypeptide-associated complex (NAC), a dynamic component of the ribosomal exit tunnel, protecting the emerging polypeptides from interaction with other cytoplasmic proteins to ensure folding and appropriate targeting of nascent protein.[41] SSB2, a molecular chaperone of Hsp70 family, binds to ribosome and plays a central role in the stabilization and folding of nascent polypeptide chains together with SSB1.[42] Deletion of SSB1/2 increased misfolding of nascent polypeptide chains in *Saccharomyces cerevisiae*.[42] STI1, as a cochaperone, has been proposed to play a critical role in the transfer of client proteins from Hsp70 to Hsp90.[43] Down-regulation of NAC-beta-1, SSB2 and STI1 under both pH 2 and/or 8 indicates the increase of unstability and misfolding of newly synthesized proteins in cytosol. As stress-repressed chaperones, down-regulation of SSB2 and NAC-beta-1 under various stresses has also been reported in *S. cerevisiae* by Albanèse et al.[42] The other three proteins, BiP, PDI and PPIase, have been proven to be the components of BiP chaperone complex in ER with the function of facilitating nascent polypeptide translocation into ER and folding.[44] Among them, PPIase, a stress-repressed chaperone reported by Albanèse et al.,[42] can accelerate the folding of proteins by catalyzing the cis−trans isomerization of proline imidic peptide bonds. BiP and PDI, as parts of quality control machinery, can recognize the misfolded proteins and remove them from the folding pathway, targeted for ER-associated degradation (ERAD).[45] Fink[46] reported that the concentration of BiP increased with elevated levels of misfolded proteins in the ER. Here, we observed that both BiP and PDI were induced by pH 2 and 8 (Figure 5B), suggesting the increase in level of misfolded protein in ER and

tendency of protein aggregation, and the importance of the two proteins in rescuing stress-denatured proteins. Subsequently, analysis on protein aggregation supported our speculation that accumulation of aggregated proteins distinctly increased at pH 2 and 8. Protein aggregation (including molecular chaperones themselves) could cause the malfunction of related pathways, and contribute to the delay of spore germination of *P. expansum*, which is worthy to be addressed in the future study. Besides the 6 proteins above, SSC1, a mitochondrial Hsp70, was also identified in the study. The proteins were down-regulated at both 5 h (pH 8) and 10 h (pH 2 and 8). Voos and Röttgers[47] indicated that SSC1 involved in the translocation of cytosolic precursor proteins to mitochondrial matrix and refolding in matrix, and repression of the protein might affect the biogenesis of mitochondrion.

Two identified proteins, PCNA and subunit 14 of TFIID in the study, are related to DNA replication and transcription, respectively. Recently, Naryzhny[48] put forward that PCNA, as a DNA sliding clamp for DNA polymerase delta, is essential for eukaryotic chromosomal DNA replication and repair. Other results demonstrated that subunit 14 of transcription initiation factor TFIID took part in the initiation of transcription by DNA-dependent RNA polymerase II.[49,50] DNA replication and transcription are fundamental processes in life cycle of cells. Though DNA replication and transcription were reported to be not essential to the early spore germination in several fungi,[2,38,51] they are required to the tube elongation during the late germination and subsequent hyphal growth.[2] Ran is a member of the Ras superfamily of small GTP-binding proteins, regulated by the GDP-GTP exchange factor (GEF) and the GTPase-activating protein (GAP) and shuttles between the nucleus and cytoplasm. Sazer[52] elucidated that Ran-GTPase system had an important role in DNA replication, nucleus protein import and export, RNA processing and export, and so on. In this study, we found that germination rates of *P. expansum* spores were markedly deceased at pH 2 and 8. Meanwhile, length of germ tube of germinated spores was significantly shorter under pH stresses. These results indicate that down-regulation of above proteins might delay the germination and affect the germ tube elongation.

Moreover, 11 identified proteins are related to metabolisms and energy pathway, including amino acid metabolism, tricarboxylic acid cycle, alcohol metabolism, fatty-acid and isoprenoid metabolism, sulfate assimilation, and cell wall metabolism, indicating the wide and complicated influences of ambient pH on metabolisms, besides fundamental biological processes.

## Concluding Remarks

In this study, we found that some critical proteins involved in protein synthesis and folding were down-regulated under both acidic and alkaline stresses in *P. expansum* spores through the 2-DE-based comparative proteomic approach, and corresponding biological processes were impaired. In addition, ambient pH affects $pH_i$ and ATP level of *P. expansum* during spore germination. Taken together, we propose that pH stress inhibits spore germination of *P. expansum* by directly changing $pH_i$ and affecting cell metabolisms and energy on one hand, and regulating the involved gene and protein expressions as a signal on the other hand. However, the pathway of signal transduction of ambient pH is needed to be addressed in the future studies. Considering the importance of protein synthesis in the early stage of spore germination, we suggest that impairing *de novo* synthesis of proteins and folding of nascent polypeptides might be the main reason. To our knowledge, this is the first report about the mechanism of ambient pH affecting fungal spore germination on the level of proteome.

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**Supporting Information Available:** Supplementary Table 1 shows MS/MS peptide sequence of the identified proteins. Supplementary Figure 1 shows inhibition of cycloheximide on spore germination of *P. expansum* spores. This material is available free of charge via the Internet at http://pubs.acs.org.

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