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Role of a major facilitator superfamily transporter in adaptation capacity of *Penicillium funiculosum* under extreme acidic stress

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ABSTRACT

Fungal species present in extreme low pH environments are expected to have adapted for tolerance to high H^+ concentrations. However, their adaptability mechanism is unclear. In this study, we isolated an acid-tolerant strain of *Penicillium funiculosum*, which can grow actively at pH 1.0 and thrived in pH 0.6. A major facilitator superfamily transporter (PfMFS) was isolated from an acid-sensitive random insertional mutant (M4) of the fungus. It encodes a putative protein of 551 residues and contains 14 transmembrane-spanning segments. A targeted mutant (M7) carrying an inactivated copy of *PfMFS* showed an obvious reduction of growth compared with the wild type (WT) and complementation of M7 with *PfMFS* restored the wild-type level of growth at pH 1.0. Further data showed that the wild-type showed higher intracellular pH than M7 in response to pH 1. Subcellular localization showed that PfMFS was a cell membrane protein. Homology modeling showed structural similarity with an MFS transporter EmrD from *Escherichia coli*. These results demonstrate that the PfMFS transporter is involved in the acid resistance and intracellular pH homeostasis of *P. funiculosum*.

1. Introduction

In nature, living organisms can be found over a wide range of extreme environments, such as high temperature, acidity/alkalinity, salinity, pressure and radiation. The organisms that thrive in extreme environments are extremophiles. They are found in all three domains of life (Archaea, Eubacteria and Eukaryotes) (Rothschild and Mancinelli, 2001; Magan, 2007). Most of extremophiles belong to the domain Archaea and the domain Bacteria, specifically to the domain Archaea. Extremophiles from the Eukarya domain can also be found living in extreme environments. Most eukaryotic extremophiles belong to the fungal and algal classes. Extremophiles play important roles in many ecosystems and provide rich microbial resources for human exploitation. The study of extremophiles has provided ground-breaking discoveries that challenge the paradigms of modern biology and make us rethink intriguing questions. The mechanisms by which different microorganisms adapt to extreme environments provide a unique perspective on the fundamental characteristics of biological processes present in most species. Extremophiles are also critical for evolutionary studies related to the origins of life. Furthermore, the application of extremophiles in industrial processes has opened a new era in biotechnology.

Acidophiles or acidophilic organisms are a kind of extremophiles which thrive under highly acidic conditions (usually at pH 2.0 or below). A strict definition is that acidophiles are organisms which are able to grow down to pH 1.0 and are able to actively grow at pH < 4.0 (Magan, 2007). Especially, acidophilic archaea and acidophilic bacteria are defined as organisms that have a pH optimum for growth of less than pH 3 (Baker-Austin and Dopson, 2007). These organisms can be found in all domain of life, including Archaea, Bacteria, and Eukaryotes. Among eukaryotic organisms, only a few species of fungi have so far been known to grow below pH 1, such as *Acontium cylatium*, *Cephalosporium* species and *Trichosporon cerebriae* (Rothschild and Mancinelli, 2001).

Organisms that live at extremely low pH are able to maintain their cytoplasm at neutral or near neutral pHs (Baker-Austin and Dopson, 2007; Bignell, 2012; Hesse et al., 2002). This intracellular pH homeostasis is achieved by a variety of mechanisms that involve restricting proton entry by the cytoplasmic membrane and purging of protons and their effects by the cytoplasm. Major categories of active pH homeostasis mechanisms include: a reversed membrane potential, highly impermeable cell membranes and proton efflux systems containing primary active transporters (such as proton ATPases) and secondary transporters (Rothschild and Mancinelli, 2001; Magan, 2007; Baker-Austin

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and Dopson, 2007; Bignell, 2012; Kanjee and Houry, 2013; Brett et al., 2011; Krulwich et al., 2011). In fungi, an ATP-dependent proton ATPase, Pam1, actively pumps protons out of the cell. Another ATP-dependent proton ATPase is the vacuolar ATPase (V-ATPase) which drives transport of protons into the vacuole (Bignell, 2012).

Among intracellular pH homeostasis mechanisms, secondary transporters have received attention because of their predominance in acidophile genome sequences (Futterer et al., 2004; Tyson et al., 2004; Crossman et al., 2004). They are believed to be an important functional adaptation of organisms to survive in an extremely acidic environment. Secondary transporters are membrane proteins that use the transmembrane electrochemical gradient of protons or sodium ions to drive transport rather than by ATP hydrolysis. Since the driving force (proton or sodium gradient) is normally directed inwards, antiport mechanisms are generally used to export substrates, while symport mechanisms operate for import. The class of secondary transporters contains many different families, but most members belong to the major facilitator superfamily (MFS) and the amino acid–polyamine–organocation (APC) superfamily. The major facilitator superfamily is the first largest superfamily of secondary transporters and includes members that function as uniporters, symporters or antiporters. The MFS superfamily consists of 74 families, each of which is usually concerned with the transport of a certain type of substrate (simple monosaccharides, oligosaccharides, amino acids, peptides, vitamins, enzyme cofactors, drugs, chromophores, nucleobases, nucleosides, nucleotides, iron chelates, and organic and inorganic anions and cations) (Reddy et al., 2012). Most MFS proteins vary between 400 and 600 amino acid residues in length and possess either 12 or 14 putative transmembrane segments (TMS) (Law et al., 2008). It has been demonstrated that some MFS proteins from fungi play a key role in resistance to natural toxic compounds and fungicides (Alexander et al., 1999; Prasad and Kapoor, 2005; Roohparvar et al., 2007; Hayashi et al., 2002; Coleman and Mylonakis, 2009; Liu et al., 2012) and virulence (Pitkin et al., 1996; Callahan et al., 1999; Coleman and Mylonakis, 2009). However, whether the MFS proteins are involved in acid resistance or intracellular pH homeostasis is unclear.

The APC superfamily appears to be the second largest superfamily of secondary transporters. The APC superfamily has been shown to include five recognized families, four of which are specific for amino acids and their derivatives (Wong et al., 2012). Currently, it has been well demonstrated that two secondary transporters of the APC superfamily, Adic and GadC, which are major components of the acid-resistance system 2 (AR2) and the acid-resistance system 3 (AR3) in *Escherichia coli*, are involved in acid resistance of *E. coli* (Richard and Foster, 2004; Foster, 2004; Ma et al., 2012; Gao et al., 2009, 2010; Kanjee and Houry, 2013). Apart from the two secondary transporters Adic and GadC, Na^+/H^+ antiporters belonging to secondary transporters from the NHE superfamily are also involved in intracellular pH homeostasis. Over-expression or deletion of a Na^+/H^+ antiporter Nhx1 in *Saccharomyces cerevisiae* resulted in alkalinization or acidification of vacuole pH, respectively (Brett et al., 2005; Ali et al., 2004). A Na^+/H^+ antiporter NhaA is essential for intracellular pH regulation in *E. coli* under alkaline conditions (Padan et al., 2005; Hunte et al., 2005; Mager et al., 2011).

Fungal species present in extreme low pH environments are expected to have adapted for tolerance to high H^+ concentrations. However, their adaptability mechanism is unclear. One important scientific question is how they adapt such an extreme low pH environment, by the above mechanisms or others? In this study, we isolated an acid-tolerant fungal strain of *Penicillium funiculosum* X33, which can grow actively at pH 1.0 and thrived in pH 0.6. We investigated the involvement of a major facilitator superfamily transporter (PfMFS) in adaptation for tolerance to high H^+ concentrations by *P. funiculosum*. Using insertional gene mutagenesis, targeted gene disruption and complementation of disrupted mutants along with cellular pH analysis, we demonstrate that the major facilitator superfamily transporter is involved in the acid resistance and intracellular pH homeostasis of *P. funiculosum*.

2. Material and methods

2.1. Strain, media and growth conditions

The fungus *P. funiculosum* X33 was originally isolated from an acidic environment in China, China vinegar with an extremely low pH 2.2. The fungus was inoculated to potato-dextrose agar (PDA: 10 g glucose, 20 g potato and 15 agar in 1 L water) plates at 28 °C and different pH or potato-dextrose broth (PDB: 10 g glucose and 20 g potato in 1 L water) medium at 28 °C and pH 1 under static culture conditions. The pH of the PDA plates and PD media was adjusted to 0.0–14.0 with H_2SO_4 and NaOH. The different pH PDA plates and PDB media were inoculated with the fungus from freshly grown PDA plates. *E. coli* DH5α was used for DNA manipulations and transformations. *Agrobacterium tumefaciens* strain LBA4404 and EHA105 were used as a T-DNA donor for fungal transformation and gene location, respectively.

To quantificationally estimate growth of *P. funiculosum* X33 at low pH, the following experiments were carried out. Growth of *P. funiculosum* were performed by inoculating 10 ml of PDB medium (pH 1.0) in a 50 ml flask with spore suspension. Growth was monitored by measuring fresh weight after culturing for 5 d at 28 °C.

2.2. DNA manipulation and sequence analysis of gene PfMFS

Standard molecular techniques were performed according to Molecular Cloning (Sambrook and Russell, 2001). Fungal DNA and RNA were extracted by the CTAB protocol or Trizol protocol, respectively. Bacterial plasmid DNA was isolated using the plasmid mini kit (OMIGA), according to the manufacturer's suggestion. Primers were designed using Primer Premier 5.0 software. The molecular mass of the mature peptide was calculated using Vector NTI 7.0 software.

2.3. Fungal isolation and growth on PDA with different pH value

The fungal strain *P. funiculosum* X33 was isolated from extreme acid conditions in our laboratory. The medium for isolation was PDA. The taxon of strain *P. funiculosum* was identified based on morphology and on rDNA sequence in the internal transcribed spacer (ITS) regions. To test the growth pH values range of the *P. funiculosum*, the strain was inoculated on PDA plates and PDB with pH values ranging from 0.5 to 13.0. The pH of the medium was regulated with H_2SO_4 and NaOH.

2.4. ATMT transformation and screening for mutants and cloning of the gene

A. tumefaciens strain LBA4404 harboring the pROK2/*hph* was used for transforming *P. funiculosum* as previously described (Han et al., 2012). The putative transformants were transferred to PDA plates containing 1000 μg/ml hygromycin B and 300 μg/ml SM. The insert of T-DNA was conformed by PCR analysis using a pair of primers (hph1/hph2) and Southern bolting with probe *hph*. The conformed transformants were then transferred to PDB media of pH 1.0, the mutants which poorly grow in pH 1.0 media were appeared after 7 days incubation in 28 °C.