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The unique role of siderophore in marine-derived *Aureobasidium pullulans* HN6.2

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Abstract The L-ornithine-N^5-monooxygenase structural gene (*SidA* gene, accession number: FJ769160) was isolated from both the genomic DNA and cDNA of the marine yeast *Aureobasidium pullulans* HN6.2 by inverse PCR and RT-PCR. An open reading frame of 1,461 bp encoding a 486 amino acid protein (isoelectric point: 7.79) with calculated molecular weight of 55.4 kDa was characterized. The promoter of the gene (intronless) was located from −1 to −824 and had three HGATAR boxes which were putative binding motifs for the respective DNA-binding motifs and one CATA box. The *SidA* gene in *A. pullulans* HN6.2 was disrupted by integrating the hygromycin B phosphotransferase (*HPT*) gene into Open Reading Frame of the *SidA* gene using homologous recombination. Of all the disruptants obtained, one strain S6 (Δ*sidA*) did not synthesize both intracellular and extracellular fusigen so that it could not inhibit growth of the pathogenic bacteria *Vibrio anguillarum* and *Vibrio parahaemolyticus*. The disruptant S6 did not grow in the iron-deplete medium and seawater medium because cell budding was stopped, but could grow in the iron-replete medium with 10 μM Fe^{3+} and Fe^{2+}. H_2O_2 in the medium was more toxic to the disruptant S6 than to its wild type HN6.2. Thus, we infer that the fusigen produced by the marine-derived *A. pullulans* HN6.2 can play a unique role in chelating, uptake and concentration of iron to maintain certain proper physiological functions within the cells and secretion of siderophore may represent an efficient tool to eliminate competitors to compete for limiting nutritional resources in marine environments.

Keywords Fusigen · Marine-derived yeasts · *SidA* gene · Gene disruption · Physiological function

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Introduction

Iron is an essential element for nearly all the organisms because it is often found in the form of heme or iron-sulfur clusters, and is used in many biochemical processes such as the reduction of atmospheric nitrogen, the synthesis of deoxyribonucleotides, respiration, the tricarboxylic acid cycle, and the synthesis of amino acids, lipids and sterols (Philpott 2006). Despite iron is the fourth most abundant element in the earth's crust, iron acquisition in marine environments is especially challenging, because this metal is most commonly found as insoluble ferric oxyhydroxides, which make its concentration only in the range from 20 pM to 1 nM in surface seawater, leading to a limited iron supply (Baakza et al. 2004; Holinsworth and Martin 2009). Many marine microorganisms including fungi produce siderophores, the

iron-chelating compounds with high affinity for ferric iron, which allow iron acquisition in spite of the low availability of Fe^{3+} ions at neutral pH. Subsequently, cells recover the iron from the ferri-siderophore complexes via specific uptake mechanisms. They are either excreted to mobilize extracellular iron or are produced intracellularly principally for iron storage and detoxification such as prevention of iron-mediated oxidative stress. Loss of the intracellular siderophore in fungi also can cause pleiotropic consequences, such as decreased growth, delayed germination of conidia, reduced asexual conidiogenesis, sexual development and virulence (Johnson 2008).

Most of fungal siderophores are hydroxamates. The biosynthesis of them usually commences with the N^5-hydroxylation of L-ornithine catalyzed by an L-ornithine-N^5-monooxygenase. This is followed by an acylation, thus forming the hydroxamate group. The assembly to the final siderophore is catalyzed by non-ribosomal peptide synthetases (NRPSs) (Haas 2003). Therefore, L-ornithine-N^5-monooxygenase is involved in the first step of fungal siderophore biosynthesis.

In recent years, many fungal species isolated from marine environments have been found to be able to produce hydroxamate or carboxylate sideophore (Holinsworth and Martin 2009) and the marine isolates produced greater quantities of siderophore than their terrestrial counterparts (Baakza et al. 2004). However, very little is known regarding genetic regulation and physiological function of siderophore in marine-derived fungi at the molecular level. In our previous studies (Wang et al. 2009a; Chi et al. 2010), *Aureobasidium pullulans* HN6.2 isolated from marine environment was found to produce 1.1 mg ml^{-1} of siderophore. The crude siderophore produced by the yeast strain HN6.2 was able to inhibit cell growth of *Vibrio anguillarum* and *Vibrio parahaemolyticus* isolated from the diseased marine animals. In another study (Wang et al. 2009b), the purified siderophore from the marine-derived *A. pullulans* HN6.2 was proved to be fusigen. The purified desferric fusigen has strong inhibition of growth of the pathogenic *V. anguillarum*. In the present study, the L-ornithine-N^5-monooxygenase structural gene (*SidA* gene) was cloned from *A. pullulans* HN 6.2 and characterized. Then, the *SidA* gene was disrupted and the effects of disruption of the gene on cell growth, siderophore synthesis and resistance to oxidative stress in the disruptant S6 (Δ*sidA*) were examined.

Materials and methods

Strains, plasmids and media

Aureobasidium pullulans HN6.2 isolated from sea saltern at Yellow Sea was found to produce 1.1 mg ml^{-1} of fusigen (Wang et al. 2009a). The marine yeast was maintained and grown in YPD medium which contained 2.0% glucose, 2.0% polypeptone and 1.0% yeast extract. The siderophore production medium (also the iron-deplete medium) contained 3.0% sucrose, 0.3% ammonium nitrate, 0.3% K_2HPO_4, 0.1% citric acid, 0.008% $MgSO_4$, 0.0002% $ZnSO_4$, 10.0 mM L-ornithine, pH 6.2 (Wang et al. 2009a). The iron-replete medium was the iron-deplete medium supplemented with 10 μM Fe_2SO_4. Artificial seawater medium was constituted of 0.147% $CaCl_2$, 0.0026% boric acid, 0.068% KCl, 1.078% $MgCl_2$, 2.35% NaCl, 0.00003% Na_2EDTA, 0.0196% $NaHCO_3$, 0.003% Na_2CO_3, 0.4% Na_2SO_4, 2.0% sucrose, 0.3% NH_4NO_3, $ZnSO_4$ 0.0002% and 10 mM L-ornithine. Natural seawater medium was made of 2.0 g sucrose and 0.3 g NH_4NO_3, $ZnSO_4$ 0.0002 g, 10 mM L-ornithine, 100 ml seawater. Gram-negative and pathogenic bacteria *V. anguillarum* and *V. parahaemolyticus* isolated from diseased marine animals were maintained in 2216E medium at 16°C (Zhang et al. 2006). The *Escherichia coli* strain used in this study was DH5α [F^- *endA1 hsdR17*(rK^-/mK^+) *supE44 thi*$^-$*1*λ^- *recA1 gyr96Δ lacU169*(*φ80lacZΔM15*)] kept in this laboratory and was grown in 5.0 ml of Luria broth (LB) in the test tube at 37°C overnight. The *E. coli* transformants were grown in 5.0 ml of LB medium with 100 μg ml^{-1} of ampicillin in the test tube at 37°C overnight. The yeast transformants were grown in HC agar (1.0% glucose, 0.3% yeast extract, 0.1% beef extract, 1.0% peptone, 0.3% malt extract, 1.5% agar, pH 5.7) containing 100 μg ml^{-1} of hygromycin B. The plasmid pCAMBIA 1381 carrying the bacterial hygromycin B phosphotransferase (*HPT*) gene was kindly supplied by Dr. Lining Zhang in Canada. pMD19-T simple for cloning of PCR products was purchased from TaKaRa Biotechnology (Dalian, China) Co., Ltd.

Isolation of DNA, restriction digestions, and transformation

Yeast genomic DNA for amplification of the gene encoding L-ornithine-N^5-monooxygenase was isolated

with TIANamp Yeast Genomic DNA Kits (TIANGEN BIOTECH (Beijing) CO., LTD.). Restriction endonuclease digestions and DNA ligations were performed according to the manufacturer's recommendations. *E. coli* was transformed with plasmid DNA according to Sambrook et al. (1989). *E. coli* transformants were plated onto LB medium containing 100 μg ml^{-1} of ampicillin.

Cloning of the full-length *SidA* gene

Inverse PCR was performed according to the methods described by Sambrook et al. (1989). The primers for inverse PCR were designed according to the partial gene encoding L-ornithine-N^5-oxygenase in *A. pullulans* HN6.2 (Wang et al. 2009b). The sense primer was LO-se: 5′-TGTCCTCCACTCGTCGCAATACTGG-3′ and the antisense primer was LO-an: 5′-TGTCAGGVG AGATATCGACCACCTC-3′. Genomic DNA was extracted from *A. pullulans* HN6.2 as described above and digested with *Pst*I. The DNA fragments were purified with TIANquick Mini Purification Kits [TIANGEN BIOTECH (Beijing) CO., LTD]. The DNA fragments were circulated by T_4 DNA ligase (MBI) at 22°C for 4 h. The circulated DNA was purified again with TIANquick Mini Purification Kits. Inverse PCR reaction system was composed of 5.0 μl of 10× La Taq buffer (TaKaRa, Japan), 8.0 μl of 2.5 mM dNTP, 1.0 μl of 20.0 μM of each primer, 1.0 μl of the circulated DNA, 0.5 μl of La Taq DNA polymerase, and 33.5 μl of double-distilled water. Touchdown PCR conditions were 94°C 3 min; 94°C 30 s, 64–0.5°C 30 s, 72°C 2.5 min, 20 cycles; 94°C 30 s, 54°C 30 s, 72°C 2 min 30 s, 15 cycles, final extension at 72°C for 15 min. The PCR products were checked and separated by agarose gel electrophoresis and sequenced. After the sequence of the PCR products was analyzed with NCBI ORF finder program and aligned with the known sequences of the genes encoding L-ornithine-N^5-monooxygenase from different yeasts by NCBI BLASTn, the full-length of L-ornithine-N^5-monooxygenase gene of *A. pullulans* HN6.2 was obtained and named *SidA* gene.

To ensure whether the *Sid*A gene was intronless, cDNA was amplified by RT-PCR. The cells of *A. pullulans* HN6.2 for RNA extraction were prepared and total RNA in the collected cells was extracted using RNAiso Reagent (TaKaRa) and the contaminated DNA was removed by DNAase I. RNA content in the sample was measured at 260 nm and the RNA amount was adjusted to 1.0 μg per 50.0 μl. The synthesis of the first strand cDNA was carried out using PrimeScript® 1st Strand cDNA Synthesis Kit (TaKaRa). The primers for RT-PCR were LoRT-se: 5′-ATGTCCCGACTGACACAATCC-3′ and LoRT-an: 5′-TCACGAA GCGAAGGTTGGAC-3′. The RT-PCR reaction was performed according to the instruction of the Kit. The RT-PCR products were purified by agarose gel electrophoresis and sequenced.

Construction of knock-out vector for disruption of the *SidA* gene

Construction of the knock-out vector designed to disrupt the *SidA* gene was carried out as described by Slightom et al. (2009) and illustrated in Fig. 1. The *TEF* promoter fragment was PCR amplified from the genomic DNA of *A. pullulans* HN6.2, using the sense primer Ts (5′-GGATCCCGACAGTTGGCTCATCA TCCGT-3′), which added a 5′ *Bam*HI site (underlined bases), and the antisense primer Ta (5′-GTTCAGGCT TTTTCATGTTTGACGGTGATGTATGGAAG-3′), which shared the sequence (underlined bases) with the *HPT*-poly(A) gene specific primer HPTs1 used in the subsequent PCR amplification. The Ts and Ta primers were designed according to the *TEF* gene sequence (accession number: U19723). The HPT-poly(A) fragment was PCR amplified from the plasmid pCAMBIA-1381 (accession number: AF234302) using the sense primer HPTs1 (5′-TCACCGTCAAACATGAAAAA GCCTGAACTCACCG-3′), which shared the seq uence (underlined bases) with the *TEF* gene specific primer Ta, and the antisense primer HPTs2 (5′-GCCGGTCGACTAATTCGGGGGATCTGGAT TTTAGT-3′), which a *Sal*I site (underlined bases) was added after four bases GCCG that were set to adjust the annealing temperature. The PCR reaction was performed in a total volume of 50.0 μl of the PCR mixture containing 5.0 μl of 10× Ex Taq buffer (Mg^{2+} Plus), 5.0 μl of 2.5 mM dNTPs, 1.0 μl of 20.0 μM each primer, 1.0 μl of the genomic DNA (10.0 ng/ml), 36.5 μl of sterile deionized water and 0.5 μl of TaKaRa Ex Taq (U/μl). The conditions for PCR amplification of the TEF fragment were as follows: pre-denaturation at 94°C for 8 min, denaturation at 94°C for 60 s, annealing temperature at 64°C for 30 s, extension at 72°C for 30 s, final extension at 72°C for 10 min. PCR was run for 30 cycles and the PCR cycler was TaKaRa PCR Thermal Cycler TP650, and the conditions for

Fig. 1 Diagram of the constructed knock-out vector

PCR amplification of the HPT-poly(A) fragment were as follows: pre-denaturation at 94°C for 8 min, denaturation at 94°C for 60 s, annealing temperature at 64°C for 30 s, extension at 72°C for 90 s, final extension at 72°C for 10 min. PCR was run for 30 cycles. The PCR products from the two PCR amplifications were mixed, denatured, allowed to anneal and subjected to a fusion PCR using the primers of Ts and HPTs2 as described above. The amplified TEF promoter-HPT-poly(A) fragments were cloned into pMD19-T simple vector (TaKaRa, Japan) and sequenced to verify the correct assembly of the insert [TEF promoter-HPT-poly(A)]. The recombinant plasmids were digested with the restriction enzymes *Bam*HI and *Sal*I and the digests were ligated into pMD19-T vector treated with the same restriction enzymes. The resulting plasmid was named pGDMIAP-1. The 5′arm of the *SidA* gene cloned above was amplified from the genomic DNA of *A. pullulans* HN6.2 using the primers Lok5–se (5′-CTGCAG TCCCGACTGACACAATCCAAAGAAG-3′) which added a *Pst*I site (underlined bases), and Lok5-an (5′-GTCGACTATGAAACCAG GTTCTGGAACCAG C-3′), which added a *Sal*I site (underlined bases). The 3′arm of the *SidA* gene was amplified from the same genomic DNA using the primer Lok3-se (5′-GGATC CCCAATTCCCCAACTCCAAAG-3′), which added a *Bam*HI site (underlined bases), and the primer Lok3-an (5′-GAATTCTCCCTGCAACCAGACACCAG-3′), which added an *Eco*RI site (underlined bases). The 5′ and 3′arm fragments were cloned into the pMD19-T simple vector, respectively, and their sequences were verified by DNA sequencing. The 5′arm fragments were then isolated from the recombinant pMD19-T simple vector after the treatment with the restriction enzymes *Pst*I and *Sal*I and ligated into pGDMIAP-1 digested with the same restriction enzymes. The resulting recombinant plasmids were named pGDMIAP-1-5′arm. The 3′arm fragments were then isolated from the recombinant pMD19-T simple vector after the treatment with the restriction enzymes *Bam*HI and *Eco*RI and ligated into pGDMIAP-1-5′arm digested with the same restriction enzymes. Finally, 5′arm-polyA-HTP-TEF-3′arm fragments (the knock-out vector, Fig. 1) were prepared using PCR with the primers Lok5–se and Lok3-an as described above.

Transformation and selection

Transformation of *A. pullulans* HN6.2 cells was done using a modified procedures described by Slightom et al. (2009). A fresh cell culture was prepared for each transformation experiment by inoculating 50.0 ml of the HC medium in a 250-ml flask with *A. pullulans* HN6.2, and allowing the organism to grow at 28°C in a shaking incubator (180 rpm) for 16–24 h. When the cell numbers in the culture reached about 2×10^7 cells ml^{-1}, 10.0 ml of the cell culture was harvested by centrifugation at 14,000×*g* for 5 min. The cell pellet was washed once with 20.0 ml of the buffer 1 (1.0 M sorbitol, 50.0 mM sodium citrate, pH 5.8). The washed cells were resuspended in 6.0 ml of buffer 1, and protoplasts were prepared by addition of 1.0 ml of 50.0 mg ml^{-1} Driselase (Sigma-Aldrich, USA) and 1.0 ml of 20.0 mg ml^{-1} Lyticase (Sigma-Aldrich, USA). The cell suspension was incubated at 22°C for 20–30 min with gentle shaking (100 rpm) in a shaking incubator, and the resulting spheroplasts were collected by centrifugation at 2,500×*g* for 5 min. The spheroplasts were washed twice, in 10.0 and 1.5 ml of the STC buffer (1.0 M sorbitol, 25.0 mM $CaCl_2$, 25.0 mM Tris/HCl, pH 7.5), respectively, and again collected by centrifugation at 2,500×*g* for 5 min. The washed spheroplasts were suspended in the STC buffer and cell concentration was adjusted to about 3.8×10^7 cells ml^{-1}. DNA transfer was accomplished by incubating 0.2 ml of the spheroplast suspension at 22°C with at least 1.0 μg of the 5′arm-polyA-HTP-TEF-3′arm fragments obtained above and 50.0 μl of the PTC solution [50% PEG 3350 (Sigma-Aldrich, P4338) in the STC buffer, 2.0 μl of *β*-mercaptoethanol]. After incubation for 5–10 min, a total of 1.0 ml of the PTC solution was added in three steps (by first adding one drop and mixing gently, after 1 min, 0.2 ml of the PTC solution was added and gently mixed with the suspension, and finally,

following yet another minute the remaining PTC solution was added, followed by gentle mixing). The transformation mixture was subsequently allowed to be incubated at 22°C for 1 h, after which the transformed spheroplasts were collected by centrifugation at 3,000×*g* and 22°C for 15 min, washed once with 1.0 ml of the 2 × HCS (Holliday complete medium containing 1.0 M sorbitol) and collected by centrifugation at 2,000×*g* for 15 min. The washed spheroplasts were suspended in 0.5 ml of the 2 × HCS and incubated at 28°C with gentle shaking (100 rpm), for 1 h, to allow regeneration of the cell walls. The cell suspension was then spread onto a two layer agar plate, with the bottom layer consisting of 1.5% agar in 12.0 ml of the HCS containing 50.0 μg ml^{-1} of hygromycin B, and the top layer consisting of 1.5% agar in 12.0 ml of HCS. The cells were then incubated at 30°C for 3–5 days and the transformed colonies generally appeared after 3 days. The putative transformants were verified by cultivation of them on the HC agar (1.5% agar in HCS) containing 100.0 μg ml^{-1} of hygromycin B. After determination of siderophore in the cells and in the supernatants of the putative transformants as described below, it was found that the transformant S6, one of them, did not synthesize any intracellular and extracellular fusigen. Therefore, the transformant S6 was used in the subsequent investigation.

Confirmation of the disrupted *SidA* gene

One loop of the cells of the yeast strain *A. pullulans* HN6.2 and the positive transformant S6 was transferred to 50.0 ml of YPD medium in 250-ml flask and aerobically cultivated for 18 h, respectively. The cells were collected and washed by centrifugation at 5,000×*g* and 4°C for 10 min. The genomic DNAs were extracted as described above and used as the template for PCR with the primers LoRT-se and LoRT-an as described above. PCR reactions and conditions were performed as described above and PCR products obtained were separated in agarose gel. The sizes of the PCR products were estimated using the Automated Documentation and Analysis System (Gene-Genius, USA). The PCR products were sequenced by Shanghai Sangon Company. The disrupted *SidA* gene was also analyzed by southern blotting as described by Watanabe et al. (2008).

Determination of siderophore

One loop of the cells of the yeast strain *A. pullulans* HN6.2 and the positive transformant S6 was transferred to 50.0 ml of the iron-deplete medium and the iron-replete medium in 250-ml flask and aerobically cultivated for 120 h, respectively. The cultures were centrifuged at 5,000×*g* and 4°C for 10 min. The supernatants obtained were used as the crude extracellular siderophore solutions. The pellets obtained were washed with sterile distilled water by centrifugation at 5,000×*g* and 4°C for 10 min and the washed cells were resuspended in distilled water. The cell suspension was submitted to repeated (200) 5s-cycles of ultrasonication using Ultrasonic Homogenier(Ninbo Scientz Biotechnology Co. Ltd) in ice bath. After disruption by ultrasonication and removal of cell debris by centrifugation, the supernatants obtained were used as the crude intracellular siderophore solutions. The iodine oxidation test (Tomlinson et al. 1971) was applied to detect the intracellular and extracellular hydroxymates siderophores. 1.0 ml of the crude siderophore solutions obtained above was hydrolyzed with 1.0 ml of 6.0 N H_2SO_4 at 130°C for 30 min to release hydroxylamines from siderophores, and 1.0 ml of this hydrolysate was buffered by adding 3.0 ml of 35% sodium acetate solution. 1.0 ml of this sample containing hydroxylamine derivative was added to 4.0 ml of potassium phosphate buffer (100 mM, pH, 7.0), 0.5 ml of 25% acetic acid solution containing 1.0% sulfanilic acid and 0.2 ml of 1.3% iodine in glacial acetic acid. After standing for 5–7 min at room temperature, the excess iodine was decolorized by addition of 0.2 ml of 0.1 N sodium thiosulfate solution. The color was developed by addition of 0.1 ml of 0.6% α-naphthylamine in 30% acetic acid and incubation for 30 min and OD value of the solution was measured at 520 nm. Calibration curves were prepared using solution of hydroxylamine hydrochloride which served as the standard. In each case the absorbance at 520 nm was measured against the appropriate reagent blank. Total protein quantity was determined using Coomassie brilliant blue assay (Bradford 1976).

Measurement of antibacterial activity

The bacterial strains used in the antibacterial assays included the pathogenic bacteria *V. anguillarum* and

V. parahaemolyticus isolated from diseased marine animals. *Vibrio anguillarum* and *V. parahaemolyticus* were grown in 2216E medium by shaking at 25°C for 10 h. Antibacterial activity of the crude siderophore solutions obtained above were performed based on the methods described by Wang et al. (2008).

Assay of hydrogen peroxide sensitivity

The cell cultures were prepared as described above and the cells in the cultures were washed by centrifugation. The washed cells were resuspended in sterile water and the final cell concentrations in the suspensions were adjusted to 10^6 cells ml^{-1}. After that, 1.0 ml of the cell suspension was incubated at 16°C for 30 min with the hydrogen peroxide concentrations from 0 to 0.13%. To determine the number of surviving cells, the suspensions were diluted 1000fold with sterile water and 0.1 ml of the dilute was plated on the iron-replete medium. After incubation for 48–72 h at 28°C, the colonies were counted and cell survival was calculated.

Results

Cloning and characterization of the *SidA* gene from *A. pullulans* HN6.2

In our previous studies (Wang et al. 2009a, b), it has been confirmed that *A. pullulans* HN6.2 produces over 1.1 mg ml^{-1} of fusigen and the purified desferric fusigen has strong inhibition of growth of the pathogenic *V. anguillarum*. Like other hydroxamates, the biosynthesis of fusigen commences with the N^5-hydroxylation of L-ornithine catalyzed by an L-ornithine-N^5-monooxygenase and this is the key step for biosynthesis of all the hydroxamates in fungi (Wang et al. 2009b). Therefore, the *SidA* gene encoding L-ornithine-N^5-monooxygenase in the marine-derived *A. pullulans* HN6.2 was cloned as described in "Materials and methods" section. The cloned *SidA* gene was found to have an open reading frame of 1,461 bp encoding a 486 amino acid protein (isoelectric point: 7.79) with calculated molecular weight of 55.4 kDa (Fig. 2). The promoter of the gene (intronless) was located from −1 to −824 and had one CATA box. The up-stream of the gene also contained three HGATAR boxes which were the putative binding motifs for the respective DNA-binding motifs. The amino acid sequence deduced from the cloned *SidA* gene had three possible N-linked glycosylation sites and the amino acids (CIGFGP, GSGQSA and DVDLVMIASGYRRD) for the putative FAD-binding, NADP-binding and substrate binding motifs.

Disruption of the *SidA* gene

As stated above, siderophore in fungi has many physiological functions (Haas 2003; Johnson 2008). Although it was found that many marine-derived fungi were able to produce different kinds of siderophore, very little is known about physiological roles of siderophore in the marine-derived fungi at molecular level (Holinsworth and Martin 2009). In order to characterize the function of the *SidA*, the ORF of the gene was disrupted by replacement with the *HPT* gene in *A. pullulans* HN6.2 as described in the "Materials and methods" section and as depicted in Fig. 1. The correct disruption was verified by PCR (Fig. 4) and southern blot analysis (data not shown). It was found that the positive disruptant S6 obtained grew on the YPD plate with hygromycin (Fig. 3B1), but did not grow in the iron-deplete medium (Fig. 3B2) while *A. pullulans* HN6.2 did not grow on the YPD plate with hygromycin (Fig. 3A1), but grew in the iron-depelete medium (Fig. 3A2). After determination of intracellular and extracellular siderophore, the results in Table 1 showed that the disruptant S6 could not synthesize both the extracellular and intracellular fusigen whereas *A. pullulans* HN6.2 could yield both. All these data demonstrated that the *HPT* gene had been expressed, all the *SidA* gene in the disruptant S6 had been deleted and the disruptant S6 obtained could not grow in the absence of iron because of its inability to synthesize any siderophore.

After PCR products were amplified from the genomic DNA of the disruptant S6 and *A. pullulans* HN6.2 using the primers LoRT-se and LoRT-an, the size (2745 bp) of the PCR products amplified from the genomic DNA of the disruptant S6 was much bigger than that (1456 bp) of the PCR products amplified from the genomic DNA of *A. pullulans* HN6.2 (Fig. 4). After sequencing of the PCR products, it was found that the PCR products amplified from the genomic DNA of the disruptant S6 contained the whole sequence of the *HPT* gene while the PCR products amplified from the genomic DNA of *A. pullulans* HN6.2 only contained the whole sequence

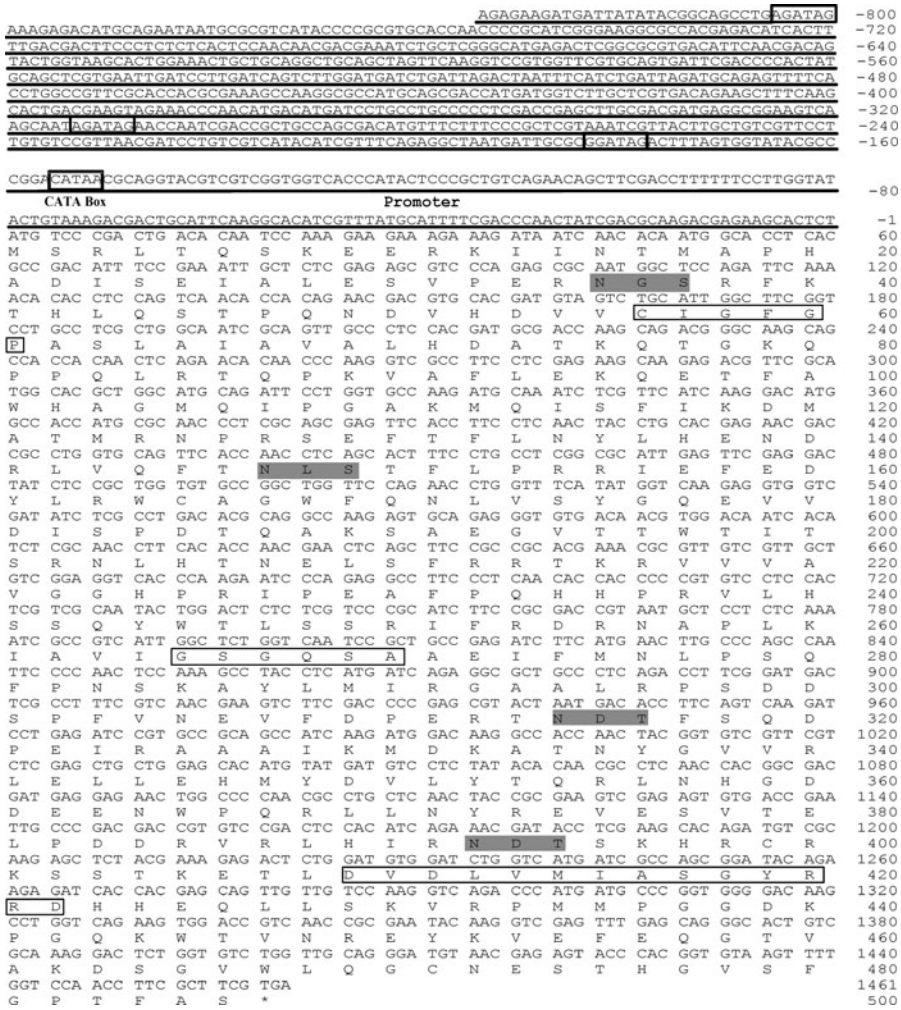

Fig. 2 The nucleotide sequence of *A. pullulans* HN6.2 L-ornithine-N^5-monooxygenase gene, its up-stream regions and deduced amino acid sequence. Translation initiation codon ATG was positioned at nt +1. Translation terminator codon TGA was located at nt +1456. The promoter region was underlined and CATA box and HGATAR binding motifs were *boxed*. Amino acid sequences for N-linked glycosylation sites were in *grey* shadow. The *boxed* amino acids (CIGFGP, GSGQSA and DVDLVMIASGYRRD) were the putative FAD-binding, NADP-binding and substrate binding motifs, respectively

of the *SidA* gene (data not shown). These results demonstrated that the *HPT* gene indeed had been inserted into the ORF of the *SidA* gene in *A. pullulans* HN6.2 so that the disruptant S6 obtained could grow on the YPD plates with hygromycin (Fig. 3B1), but could not synthesize any siderophore (Table 1).

Effects of different concentration of Fe^{2+} and Fe^{3+} on cell growth of the disruptant S6 and *A. pullulans* HN6.2

The results in Fig. 3 have shown that the disruptant S6 could not grow in the iron-deplete medium while *A. pullulans* HN6.2 could. However, both the disruptant S6 and its wild type HN6.2 grew well in the iron-deplete medium supplemented with 10 μM Fe^{2+} or Fe^{3+} (Fig. 5a–d), indicating that Fe^{3+} was reduced to Fe^{2+} and Fe^{2+} was taken up for cell growth by a siderophore-independent ferrous uptake system in them.

When they were incubated in the iron-deplete medium supplemented with 1.5 mM Fe^{2+} or Fe^{3+}, both of them could grow in the iron-deplete medium supplemented with 1.5 mM Fe^{2+} (Fig. 5e, f), but both of them could not grow in the iron-deplete medium supplemented with 1.5 mM Fe^{3+} (Fig. 5g, h).

As the yeast strains were isolated from marine environment, their growth in the artificial seawater medium and natural seawater medium were examined. It could be observed from the results in Fig. 5i–l that when they were incubated in the artificial seawater medium and natural seawater medium, respectively, which were also deficient in iron (Holinsworth and Martin 2009), only its wild type HN6.2 could grow on such media, but the disruptant S6 could not.

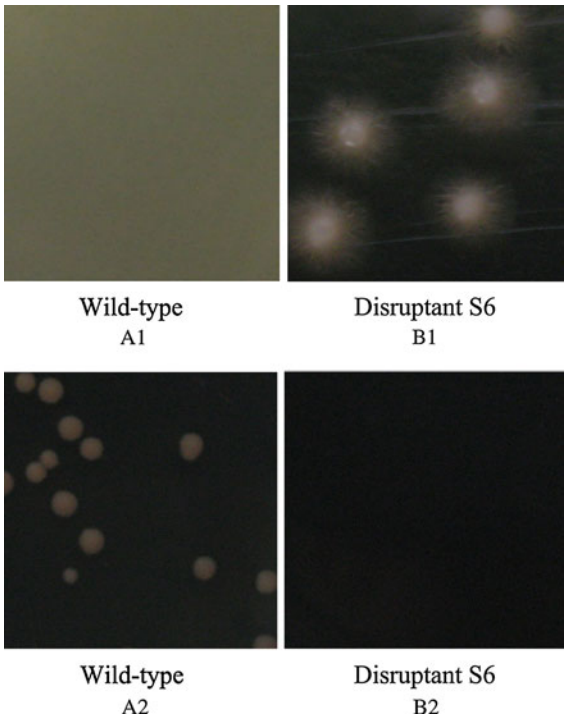

Fig. 3 Cell growth of wild-type strain HN6.2 and its disruptant strain S6 on YPD solid medium supplemented with hygromycin B (**A1**, **B1**) and on the iron deplete solid medium (**A2**, **B2**). Each colony originated from one single yeast cell. The incubation temperature and time were 28°C and 48 h, respectively

Cell budding of the disruptant S6 and its wild type HN6.2

When the disruptant S6 and its wild type HN6.2 were aerobically cultivated in the iron-deplete medium, respectively, the wild type strain HN6.2 budded normally (Fig. 6a), but the disruptant S6 stopped to bud (Fig. 6b). However, when the disruptant S6 and its wild type HN6.2 were aerobically grown in the iron-deplete medium supplemented with 10 μM Fe^{2+}, respectively, both the wild type strain HN6.2 and the disruptant S6 budded normally (Fig. 6c, d). It also could be seen clearly from Fig. 6 that the size of the budding cells was much bigger than that of the non-budding cells incubated in the iron-deplete medium.

Antibacterial activity

As discovered in our previous studies (Wang et al. 2009a, b), the fusigen produced by *A. pullulans* HN6.2 had strong inhibition of growth of *V. anguillarum* (Fig. 7a) and *V. parahaemolyticus* (Fig. 7d) which were the common pathogens in marine animals. However, after disruption of the *SidA* gene, the supernatants produced by the disruptant S6 had no ability to inhibit cell growth of *V. anguillarum* (Fig. 7b) and *V. parahaemolyticus* (Fig. 7c).

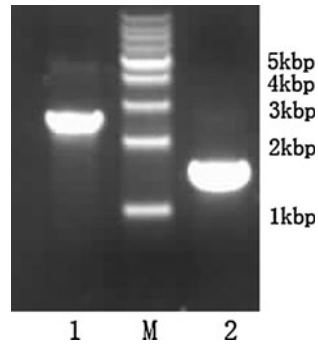

Fig. 4 The PCR products amplified from the genomic DNA of the disruptant S6 and *A. pullulans HN6.2. 1* the disruptant S6 (2745 bp), *M* 1 Kbp DNA Marker, *2 A. pullulans* HN6.2 (1456 bp)

Table 1 Concentration of siderophores (mM) produced by the wild type strain HN6.2 and its disruptant S6

Strains	Hydroxymates siderophores	
	Extracellular	Intracellular
HN6.2	0.44 ± 0.02 mM	4×10^{-6} ± 0.2 mM/mg. protein
S6	0	0

Data are given as means ±SD, n = 3

Sensitivity to H_2O_2 stress

Many results have shown that deficiency of siderophore in filamentous fungi causes very sensitivity to H_2O_2 stress because free iron acts as a catalyst in several oxygen radical reactions which generate toxic side products (Hof et al. 2009). However, very little is known about the roles of siderophore in marine-derived yeasts during H_2O_2 stress treatment. Therefore, the cell survival of *A. pullulans* HN6.2 and the disruptant S6 was examined during H_2O_2 stress treatment as described in "Materials and methods" section. As expected, the disruptant S6 was much more sensitive to H_2O_2 stress than *A. pullulans* HN6.2, especially when H_2O_2 concentration was higher than 0.04% (Fig. 8).

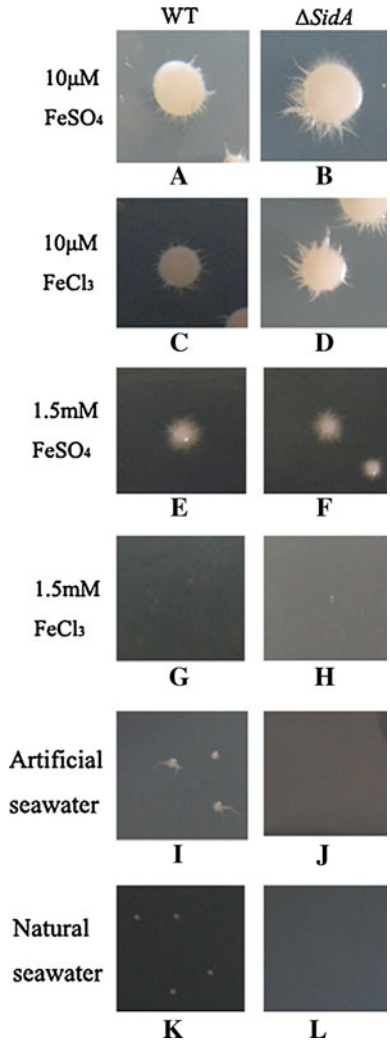

Fig. 5 The cell growth of the disruptant S6 (**a**, **d**, **f**, **h**, **j**, **l**) and its wild type HN6.2 (**a**, **c**, **e**, **e**, **g**, **i**, **k**) on different media. *a and b* the iron-deplete medium supplemented with 10 μM $FeSO_4$, *c and d* the iron-deplete medium supplemented with 10 μM $FeCl_3$, *e and f* the iron-deplete medium supplemented with 1.5 mM $FeSO_4$, *g and h* the iron-deplete medium supplemented with 1.5 mM $FeCl_3$, *i and j* the artificial seawater medium, *k and l* the natural seawater medium. Each colony originated from one single yeast cell. The incubation temperature and time were 28°C and 48 h, respectively

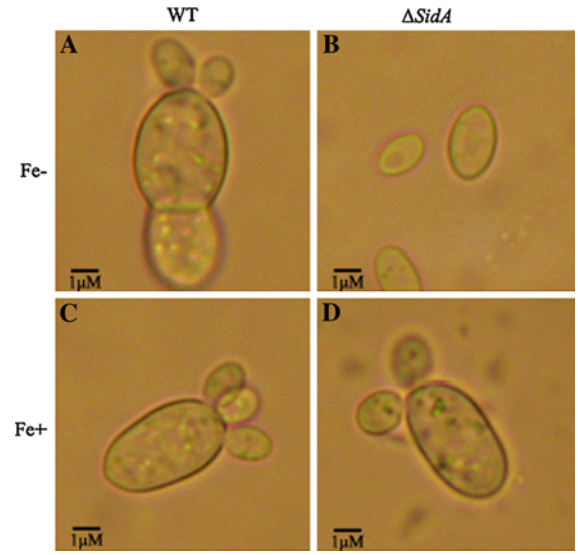

Fig. 6 Cell budding of the disruptant S6 (**b**, **d**) and its wild type HN6.2 (**a**, **c**) grown in the iron-deplete medium (**a**, **b**) and the iron-replete medium (**c**, **d**)

Discussion

The size of the cloned *SidA* gene from *A. pullulans* HN6.2 was 1,461 bp encoding a 486 amino acid protein (isoelectric point: 7.79) with calculated molecular weight of 55.4 kDa (Fig. 2) and the gene had no intron. The promoter of the gene contained three HGATAR boxes which were the putative binding motifs for the respective DNA-binding motifs so that the fusigen biosynthesis in this organism was greatly repressed in the presence of iron (Wang et al. 2009b). In contrast, the promoter of *Ustilago maydis sid1* only has one GATA box and that of the *affA* gene from *Aspergillus oryyzae* has several GATA boxes (Yamada et al. 2003). However, the ATCWGATAA motif existed in the promoters of *U. maydis sid1* (siderophore biosynthesis), *Schizosaccharomyces pombe str1* (siderophore transporter) and *S. pombe fio1-fip1* (reductive iron assimilatory gene cluster) was not found in the promoter of the *SidA* gene cloned in this study (Schrettl et al. 2008) (Fig. 2). It has been reported that there is a long sequence encoding an *A. pullulans* gene cluster consisting of *SidA*, a NRPS and an ABC transporter: APU85909/U85909 (http://www.ncbi.nlm.nih.gov/nuccore/4099310) and the genes encoding ornithine monooxygenase and ferrichrome NRPS are clustered in *U. maydis* (Haas 2003). However, it is still unknown if this happened in *A. pullulans* HN6.2 used in this study.

The amino acid sequence deduced from the cloned *SidA* gene had the amino acids (CIGFGP, GSGQSA and DVDLVMIASGYRRD) for the putative FAD-binding, NADP-binding and substrate binding motifs. The complete gene of L-ornithine N^5-monooxygenase in *A. oryzae* includes a 1575 bp ORF, one 66-bp intron, encodes 502 amino acids with putative FAD-binding,

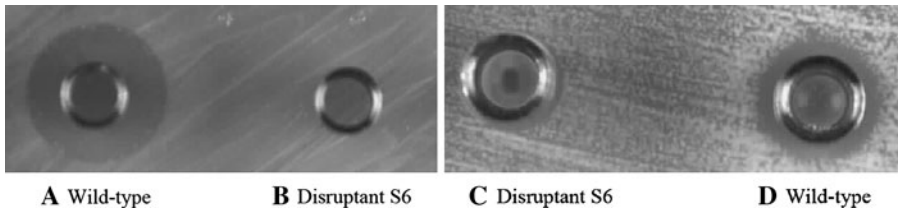

Fig. 7 Antibacterial activity of the supernatants produced by *A. pullulans* HN6.2 and the disruptant S6. **a** Antibacterial activity (of the supernatant produced by *A. pullulans* HN6.2) against *V. anguillarum.* **b** No antibacterial activity (of the supernatant produced by the disruptant S6) against *V. anguillarum.* **c** No antibacterial activity (of the supernatant produced by the disruptant S6) against *V. parahaemolyticus.* **d** Antibacterial activity (of the supernatant produced by *A. pullulans* HN6.2) against *V. parahaemolyticus*

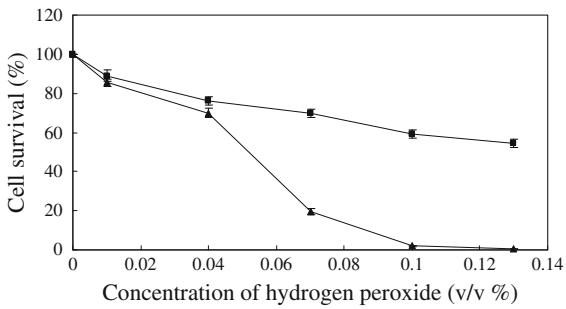

Fig. 8 Cell survival of *A. pullulans* HN6.2 (*filled square*) and the disruptant S6 (*filled triangle*) during H_2O_2 stress treatment. Data are given as means ±SD, $n = 3$

NADP-binding and substrate binding motifs (Yamada et al. 2003). In fact, all the genes of L-ornithine-N^5-monooxygenase in *U. maydis*; *Pseudomonas aeruginosa*, *Aspergillus nidulans* and *S. pombe* have such binding motifs (Labbe et al. 2007; Yamada et al. 2003). This demonstrated that the cloned *SidA* gene from *A. pullulans* HN6.2 indeed encoded L-ornithine-N^5-monooxygenase. The degree of identity between the L-ornithine-N^5-monooxygenase obtained in this study and that in *Aspergillus fumigatus* (XP_755103), *A. pullulans* (U85909), *Aspergillus flavus* (XP_002378181), *Neurospora crassa* (XP_960300), *Penicillium marneffei* (XP_002146453), *U. maydis* (P56584) was 43.9, 83.9, 45.6, 41.2, 40.5 and 24.0%, respectively, (data not shown), indicating that the L-ornithine-N^5-monooxygenase in *A. pullulans* HN6.2 was the most closely related to that in *A. pullulans* (U85909).

Because *A. pullulans* HN6.2 synthesized only fusigen (Table 1), after deletion of the gene, the disruptant S6 obtained could not grow in the iron-deplete medium (Fig. 3). In contrast, many fungal strains have been shown to simultaneously synthesize more than one type of hydroxamate siderophore (Renshaw et al. 2002). For example, the plant pathogenic fungus *Magnaporthe grisea* excretes siderophores of the coprogen-type for iron acquisition and uses ferricrocin for intracellular iron storage and deletion of the *M. grisea SSM2* gene, which encodes a NRPSs, resulted in a loss of the production of all the coprogens, but ferricrocin production was not affected. Upon deletion of *M. grisea OMO1*, a gene predicted to encode an L-ornithine-N^5-monooxygenase, no siderophores of any type were detected (Hof et al. 2009). Disruption of the *sidA* gene encoding the first committed step in siderophore biosynthesis in *A. nidulans* also led to a complete loss of excreted and cellular siderophores (Eisendle et al. 2003). In contrast, most siderophore-producing fungi employ a ferrichrome-type intracellular siderophore (Haas 2003).

The results in Fig. 5 showed the disruptant S6 and its wild type HN6.2 grew well in the iron-deplete medium supplemented with 10μM Fe^{2+} or Fe^{3+}. This indicated that the marine yeast had a second high-affinity iron uptake system, most likely reductive iron assimilation, which is present in most fungi (Haas et al. 2008), and took up Fe^{2+} by a siderophore-independent ferrous uptake system. In fact, the yeast *S. pombe* also has one pathway for acquisition of iron by production, excretion, and capturing of siderophore–iron complexes and a second pathway which requires enzymatic reduction of ferric iron at the cell surface prior to uptake by a permease–oxidase complex (Labbe et al. 2007). Upon deletion of *M. grisea OMO1*gene, the growth of Domo1 mutant was very poor when iron was lacking or at concentrations of 10 μM $FeCl_3$ and $FeSO_4$, however, growth rates were nearly the same as those of wild-type controls when the medium contained 100 μM or 1.5 mM $FeCl_3$, $FeSO_4$ or ferric citrate (Hof et al. 2009). Deficiency in *sidA* causes the complete absence of siderophores in

A. nidulans and *A. fumigatus*. *A. nidulans* Δ*sidA* mutants are unable to grow unless supplemented with siderophores or high amounts of ferrous iron. In contrast, *sidA*-deficient *A. fumigatus* strains are viable due to the presence of a reductive iron assimilatory system (Eisendle et al. 2006). This demonstrated that the demand on iron for growth of the disruptant S6 obtained in this study was different from that of Domo1 mutant of *M. grisea*, *A. fumigatus* and *A. nidulans* Δ*sidA* mutants.

The marine yeasts used in this study grew in the iron-deplete medium supplemented with 1.5 mM Fe^{2+} (Fig. 5e, f), but did not grow in the iron-deplete medium supplemented with 1.5 mM Fe^{3+} (Fig. 5g, h), suggesting that the ferric reductase activity responsible for reduction of Fe^{3+} was inhibited in the presence of 1.5 mM Fe^{3+}, but Fe^{2+} was still taken up for cell growth by them. These results also indicated that ferric iron was more toxic than ferrous iron to *A. pullulans*. In contrast, on a medium containing 1.5 mM $FeSO_4$, which is 150-times the concentration of a standard *Aspergillus* growth medium, the growth of the *sidA* mutant of *A. nidulans* was only partially restored, whereas the addition of $FeCl_3$ or ferric citrate to a concentration of 1.5 mM had no effect (Eisendle et al. 2006). The inhibition of growth under the same condition in the presence of the ferrous iron chelator bathophenanthroline disulfonate was also found in *A. fumigatus* (Schrettl et al. 2007). This meant that the disruptant S6 obtained in this study demanded a much lower Fe^{2+} concentration to restore the growth than the *sidA* mutant of *A. nidulans* did.

The results in Fig. 5i–l revealed that only its wild type HN6.2 could grow in the seawater media, but the disruptant S6 could not. This meant that the disruptant S6 did not chelate, take up and concentrate iron from the media deficient in iron because of lack of siderophore (Table 1). As the very low availability of Fe^{3+} ions in the marine environments (Holinsworth and Martin 2009), we think that the fusigen produced by the marine-derived *A. pullulans* HN6.2 in marine ecosystem may play unique roles in its cell growth and chelating, uptake and concentration of iron. This is the first time to report the physiological roles of siderophore in marine-derived yeasts.

Figure 6 showed that the wild type strain HN6.2 grown in the iron-deplete medium budded normally, but the disruptant S6 grown in the same medium stopped to bud. It has been reported that after deletion of the *M. grisea SSM2* gene, the mutant strains (Dssm2 mutants) produced fewer conidia and upon deletion of *M. grisea OMO1*gene, the strain (Domo1 mutant) was aconidial (Hof et al. 2009). This suggests that asexual reproduction in both yeasts and filamentous fungi is negatively affected after deletion of the gene encoding L-ornithine-N^5-monooxygenase.

Deficiency in siderophore in the disruptant S6 caused loss of its ability to inhibit growth of the pathogenic bacteria (Fig. 7). Therefore, in marine environment, like killer toxins produced by some marine yeasts (Buzdar et al. 2011; Chi et al. 2010), secretion of siderophore may also represent an efficient tool to eliminate competitors to compete for limiting nutritional resources. It also has been reported that the siderophores produced by *A. fumigatus*, *Alternaria* sp., *Fusarium* sp. are also involved in plant and animal virulence (Haas 2008). In our previous study (Wang et al. 2009a, b), we thought that the fusigen produced by the yeast strain HN6.2 had much higher affinity for iron than that produced by the pathogenic bacteria used in this study, leading to the strong growth inhibition of the bacteria by depriving the bacteria of iron as *V. anguillarum* also can produce its own siderophores, anguibactin.

In the presence of H_2O_2 more than 0.04% (v/v), cell survival of the disruptant S6 was lost more quickly than that of *A. pullulans* HN6.2 (Fig. 8). However, the minimal inhibitory concentrations of the oxidative stress reagent H_2O_2 for wild-type, Domo1 and Dssm2 of *M. grisea* were >16, 12 and 16 mM, respectively, (Hof et al. 2009). Lack of the intracellular siderophore in *A. nidulans* also causes increased sensitivity of conidia to oxidative stress, but not hyphae (Eisendle et al. 2006). This indicates that siderophore in both yeast cells and fungal cells, including marine eucaryotic microorganisms is responsible for their resistance to oxidative stress.

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