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Chemical and biological characterization of siderophore produced by the marine-derived *Aureobasidium pullulans* HN6.2 and its antibacterial activity

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Abstract After analysis using HPLC and electronic ion spray mass spectroscopy, the purified siderophore produced by the marine-derived *Aureobasidium pullulans* HN6.2 was found to be fusigen. The purified desferric fusigen still had strong inhibition of growth of the pathogenic *Vibrio anguillarum* while the fusigen chelated by Fe^{3+} lost the ability to inhibit the growth of the pathogenic bacterium. The added iron in the medium repressed expression of the hydroxylase gene encoding ornithine N^5-oxygenase that catalyzes the N^5-hydroxylation of ornithine for the first step of siderophore biosynthesis in the yeast cells while expression of the hydroxylase gene in the yeast cells grown in the medium plus ornithine was enhanced.

Keywords Siderophore biosynthesis · Fusigen · *A. pullulans* · Ornithine · Fe^{3+}

Introduction

Siderophores are low molecular weight, iron-chelating ligands produced by nearly all the microorganisms during extreme iron-depleted conditions for the solubilisation of extracellular ferric iron (Wang et al. 2009; Li et al. 2008). So far, among microorganisms, only strictly anaerobic bacteria and some yeasts such as *Saccharomyces cerevisiae*, *Candida albicans*, and *Cryptococcus neoformans* have been found not to make siderophore (Johnson 2008). However, these yeasts can utilize siderophores produced by others. The most significant feature of siderophores is their extremely high affinity for ferric ion. Siderophores can affect microorganisms in the environments in several ways as a result of their role as iron-scavenging compounds, especially marine microorganisms because iron is an essential nutrient for virtually all forms of life and is difficult to obtain due to its low solubility in marine environments (Baakza et al. 2004). In fungal cells, siderophores are required for virulence, resistance to oxidative stress, asexual/sexual development, iron storage, protection against iron-induced toxicity in some fungal organisms and fungal–host interactions (Johnson 2008). Siderophores are also found to have many applications in medical industry, agricultural industry, and environmental sciences (Li and Chi 2004; Renshaw et al. 2002). For example, they can be used to control growth of the pathogenic bacteria in marine fish in marine environments and the complexing ability of siderophores can be used to develop processes for metal recovery or remediation of waste sites, including radioactive waste as they are extremely effective at solubilizing actinides and other metals from polluted environments.

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It has been confirmed that yeasts produce only hydroxamate-type compound (Riquelme 1996). Hydroxamate siderophores are derived from the non-proteinogenic amino acid ornithine and can be classified into four structural families: fusarinines, coprogens, ferrichromes, and rhodotorulic acid (Johnson 2008).

In our previous studies (Wang et al. 2009; Chi et al. 2009), over 300 yeast strains isolated from different marine environments were screened for their ability to produce siderophore. Among them, only the yeast strain HN6.2 which was identified to be *Aureobasidium pullulans* was found to produce high level of the siderophore. Under the optimal conditions, this yeast strain could produce 1.1 mg ml^{-1} of the siderophore. The crude siderophore produced by the yeast strain HN6.2 was able to inhibit cell growth of *Vibrio anguillarum* and *V. parahaemolyticus*, isolated from the diseased marine animals. However, structures of these siderophores produced by *A. pullulans* HN6.2 have not been elucidated so far. Hence, in the present study, the siderophore was purified and characterized and the antibacterial activity of the purified siderophore was determined.

Materials and methods

Microorganisms

A. pullulans HN6.2 isolated from sea saltern at Yellow Sea was found to produce 1.1 mg ml^{-1} of siderophore (Wang et al. 2009). Gram-negative and pathogenic bacterium *V. anguillarum* isolated from diseased marine animals was maintained in 2216E medium at 16°C (Zhang et al. 2006).

Preparation of the crude siderophore solution

One loop of the cells of the yeast strain HN6.2 was transferred to 50.0 ml of the siderophore production medium in 250 ml flask and aerobically cultivated for 20 h. Five milliliters of the cell suspension ($OD_{600nm} = 20.0$) was transferred to 45 ml of the same production medium which 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 in each flask (Wang et al. 2009). The flask was shaken at 28°C and 180 rpm for 120 h. The culture was centrifuged at $10,000 \times g$ and 4°C for 10 min and the amount of the siderophore in the supernatant was determined as described below. The supernatant obtained was filtered on a 0.22 μm Millipore filter and the filtrate was used as the crude siderophore solution (sterile).

Quantitative determination of siderophore

The amount of siderophore in the supernatant was measured quantitatively at 440 nm by using a spectrophotometer, and deferoxamine mesylate (one type of hydroxamates) from Sigma served as standard (Atkin et al. 1970). Five hundreds of microliters of the supernatant obtained were added to 2.5 ml of the solution (pH 2.0) which contained 5.0 mM $FeCl_3$ and 0.1 M $HClO_4$ and the mixture was incubated at room temperature for 30 min. After that, the OD value of the mixture was read spectrophotometrically at 440 nm. A standard curve was prepared with deferrioxamine mesylate (standard hydroxamate) using the same $FeCl_3$ method. The deferrioxamine mesylate solution contained 0, 0.05, 0.1, 0.25, 0.5, 0.75 and 1.0 mg/ml of deferoxamine mesylate, respectively. The amount of siderophore in the supernatant was extrapolated from the standard curve of deferoxamine mesylate.

Extraction of siderophore

The siderophore was extracted from the crude siderophore solution according to the methods described by Neilands (1984). The crude siderophore was lyophilized and the lyophilized sample was dissolved in distilled water. The solution obtained was supplemented with $FeCl_3 \cdot 6H_2O$ and without $FeCl_3 \cdot 6H_2O$, respectively and the solution with $FeCl_3 \cdot 6H_2O$ was stirred until a brown color developed. pHs of the colored solution and the solution without $FeCl_3 \cdot 6H_2O$ were adjusted to 3.0 using 1.0 N H_2SO_4 and the powdered ammonium sulfate was dissolved in the solutions until the saturation was 50% in order to remove the proteins. The siderophore in the solutions was extracted into benzylalcohol and the extract was filtrated using filter paper and the filtrate was mixed with three volumes of ether. The siderophore was extracted into three volumes of distilled water. Four volumes of ethanol were added to the extract and maintained at 4°C overnight. The polysaccharide precipitate formed was removed by

centrifugation at 14,000×*g* and the residual ethanol in the supernatant obtained was evaporated at 60°C. The residual ethanol and benzylalcohol was completely removed by extraction with ether. Finally, the solution was concentrated to several milliliters on a vaccum rotary evaporator at 37°C. The solid particle in the concentrate was removed by filtration (3 kDa cut-off) at room temperature through a LabscaleTM TFF System and the filtrate was used as the partially purified siderophore and stored at 4°C.

HPLC separation and determination

The solution of the partially purified siderophore was further concentrated to several milliters on a vaccum rotary evaporator at 37°C and the siderophore in the concentrate was absorbed onto Sephadex LH-20 column and washed with three volumes of distilled water and desorbed with one volume of methanol. The elute was analyzed by the reversed-phase HPLC (YMC-Pack ODS (A), 20 × 250 mm, 5 μm, 1.0 ml/min; YMC, Japan) and was separated on a semipreparative C_{18} reversed-phase column using a gradient of methanol : H_2O (5–100%). The separated siderophore was collected, and lyophilized and analyzed by the reversed-phase HPLC for determination of its purity. The HPLC conditions: flow rate was 1.0 ml/min; column temperature was 30°C; the sample volume was 80 μl; detector: waters996 Diode-Array Detector; detect wavelength: 201 nm; sensitivity: 0.02 AUFS.

Electronic ion spray mass spectroscopy (ESI-MS)

ESI-MS was measured on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. A small amount of the purified siderophore obtained above was dissolved in 5.0% acetonitrile. The siderophore in the solution was analyzed by the mass spectroscopy. The conditions are: source temperature 80°C; desolvation temperature was 200°C; Capillary voltage: 3,000 V.

Antimicrobial assays

Gram-negative and pathogenic bacterium (in marine animals) *V. anguillarum* was aerobically grown in the 2216E medium (polypeptone 5.0 g, yeast extract 1.0 g, FePO4 0.01 g, aged seawater 1.0 l, pH 7.6) by shaking at 25°C for 10 h. The culture was harvested and washed by centrifugation at 5,000 rpm at 4°C with sterile saline water. The washed cells were resuspended in the sterile saline water and 100 μl of the suspension was spread on the plates of the 2216E medium with 2.0% agar. About 6-mm-diameter sterile Oxford-cups (6 mm × 10 mm) were put on the plates. Finally, 200 μl of the purified siderophore solution (sterile) and crude siderophore solution (sterile) was added to each cup and incubated at 25°C for 72 h and the diameter of the inhibition zone was used as a measure of the inhibitory activity of the siderophore solution. The standard siderophore solution with 10 mg ml^{-1} of deferoxamine mesylate was used as positive control (Wang et al. 2007).

Total RNA isolation and RT-PCR

Total RNA was isolated from the yeast strain HN6.2 grown in the production medium without and with iron or L-orithine supplementation using RNAiso Reagent (TaKaRa, Japan) according to the manufacturer's instructions. Total RNA in the sample was determined by spectrophotometry at 260 nm. The same amount of total RNA (1.0 μg) was used to analyze mRNA levels by RT-PCR. To synthesize the first cDNA strand of the gene encoding the hydroxylase responsible for the N^5-hydroxylation of ornithine, the first step in the fungal siderophore biosynthesis, the reverse transcriptase M-MLV (RNase H^-) was used according to the protocols provided by the manufacturer. PCR amplification was performed using Taq DNA polymerase from Promega. One set of the specific primers, the forward primer S: 5′-AGTTCACCAACCTCAGCA CTTTCC-3′ and the reverse primer A: 5′-ACCAG AGCCGATGACAGCGAT-3′, designed according to the sequence of the gene encoding the hydroxylase (the accession number: U85909) were used for RT-PCR. One set of the specific primers for amplification of the 18S rRNA gene (the accession number: FJ023536) (the forward primer S1: 5′-GTCGGGGAA CCAGGACTTTTACTT-3′ and the reverse primer A1: 5′-AGGTGCCGAGCGAGTCAAAAT-3′) was designed according to the sequence of the 18S rRNA gene in this yeast strain. The conditions for RT-PCR amplification were as follows: initial denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing temperature at 51°C for 30 s, extension at 72°C for 1 min and final extension at 72°C for 10 min.

RT-PCR was run for 32 cycles. The 18S rRNA gene was used as an internal standard.

Results

Isolation and purification of the siderophore

After the partially purified siderophore was flowed through Sephadex LH-20 column, the elute was analyzed using the reversed-phase HPLC. The results in Fig. 1 show that there was one main sharp peak at 17 min. The component of the peak was regarded as the target compound. Then, the elute was further separated on a semipreparative C_{18} reversed-phase column as described in experimental procedures. The separated siderophore was collected and analyzed by the reversed-phase HPLC. The results in Fig. 2 indicate that there was only one single sharp peak. This means that the purified compound was obtained.

Analysis of ESI-MS

The main peak isolated by semi-preparative HPLC was subsequently analyzed by ion spray mass spectroscopy (Fig. 3). The observed quasi-molecular ion peaks (MH+ = *m/z* 780.9; M2H 2+ = *m/z* 391) were characteristic of fusigen (779 Da).

In order to further obtain the evidence that the purified siderophore is fusigen. The purified siderophore was analyzed by using high-resolution ESI-MS. The results in Fig. 4 reveal that the mass of the molecular ion peak ($\triangle G + H^{+1}$) was 780.2963 whereas the calculated mass was 780.2993. The ppM value between them was −3.8. Therefore, the molecular formula was $C_{33}H_{52}O_{12}N_6Fe$. All the results were identical with those from Haselwandter et al. (1992). Finally, the chemical structure of the purified siderophore was determined and shown in Fig. 5.

Antibacterial activity of the purified siderophore

In our previous studies (Wang et al. 2009), it was found that the crude siderophore produced by the yeast strain HN6.2 was able to greatly inhibit cell growth of *V. anguillarum* isolated from the diseased marine animals. Therefore, antibacterial activity of the purified siderophore with Fe^{3+} and without Fe^{3+} against the marine animal pathogen, *V. anguillarum*

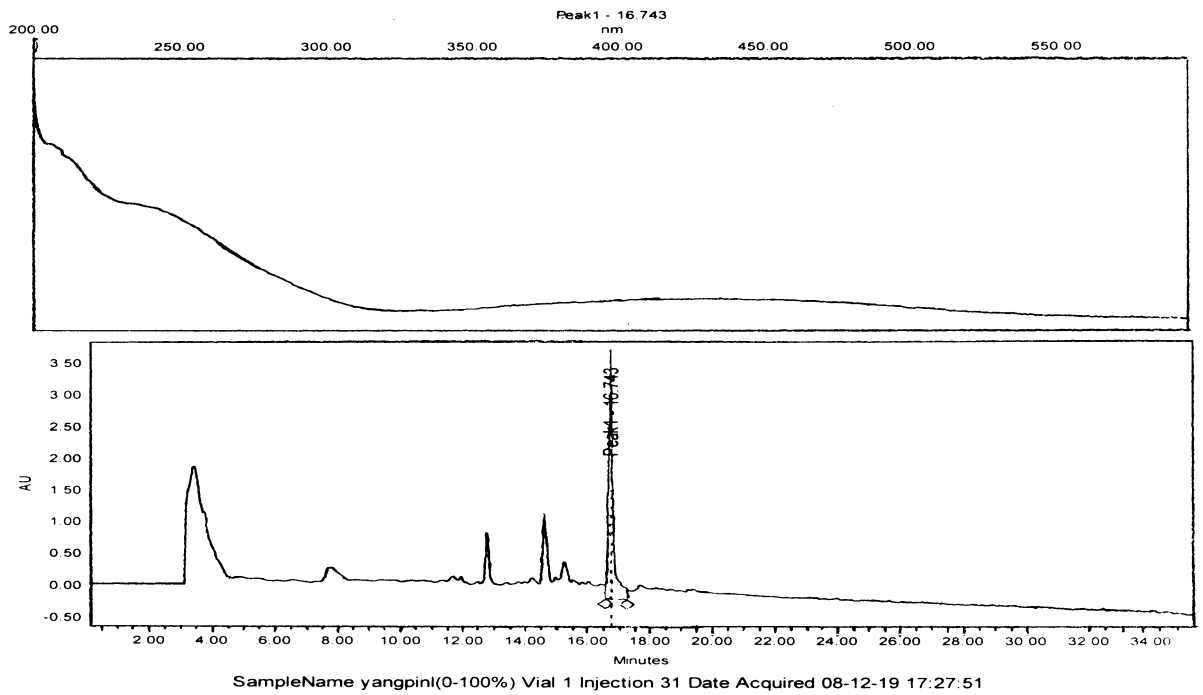

Fig. 1 HPLC analysis of the elute from SephadexLH-20 column

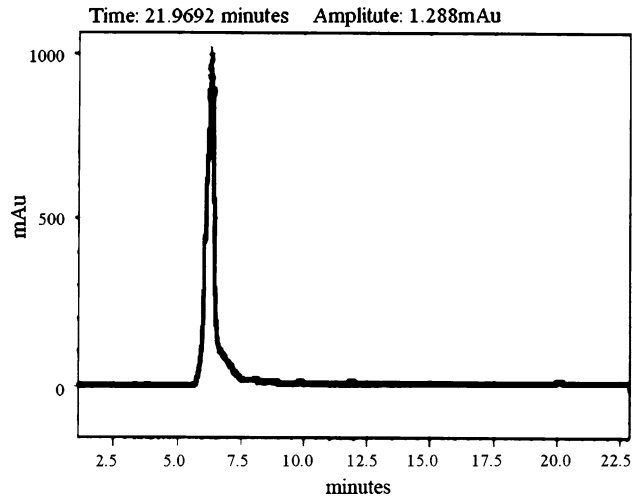

Fig. 2 HPLC analysis of elute from a semipreparative C_{18} reversed-phase column

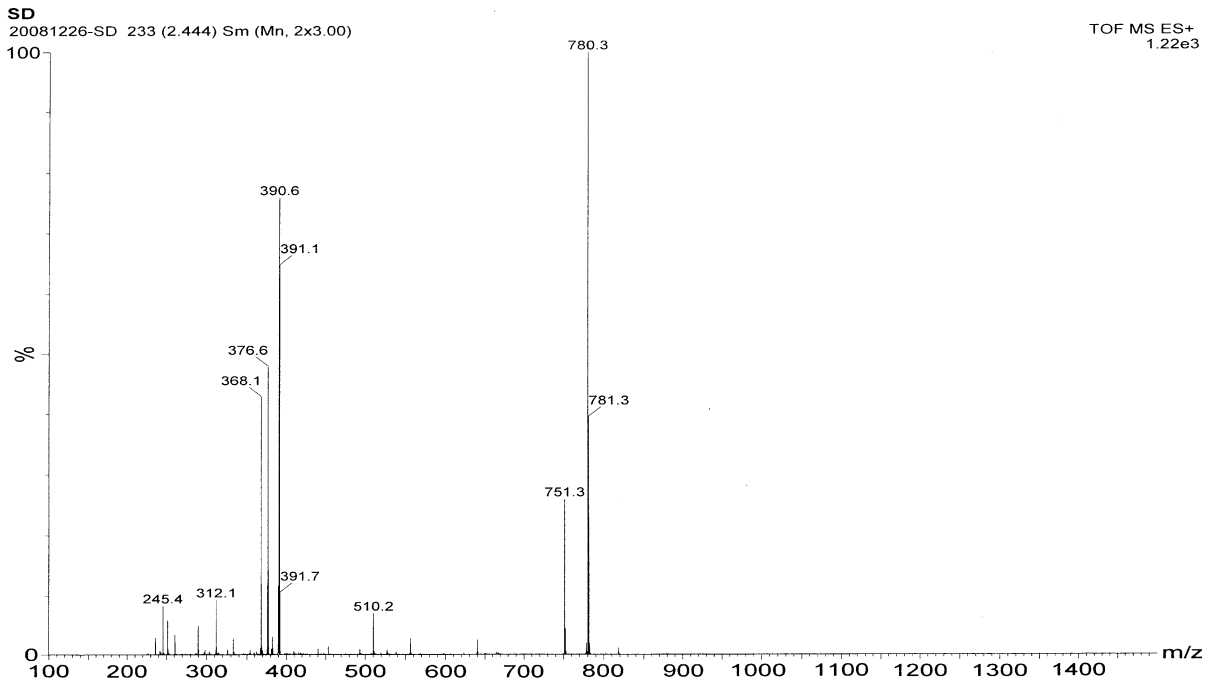

Fig. 3 The ESI-MS spectra of the purified siderophore

was determined. It can be noted from the results in Fig. 6 that only the crude siderophore and the purified siderophore without Fe^{3+} had the ability to inhibit the cell growth of the marine animal pathogen, *V. anguillarum* (Fig. 6a, b) while the purified siderophore chelated by Fe^{3+} lost its ability to inhibit the cell growth of the marine animal pathogen, *V. anguillarum* (Fig. 6c, d).

Effects of added ornithine and Fe^{3+} on the hydroxylase gene expression

The first committed step in siderophore biosynthesis is the N^5-hydroxylation of ornithine catalyzed by ornithine N^5-oxygenase (Haas 2003). In our previous studies (Wang et al. 2009), it was found that ornithine has stimulatory effects on siderophore production

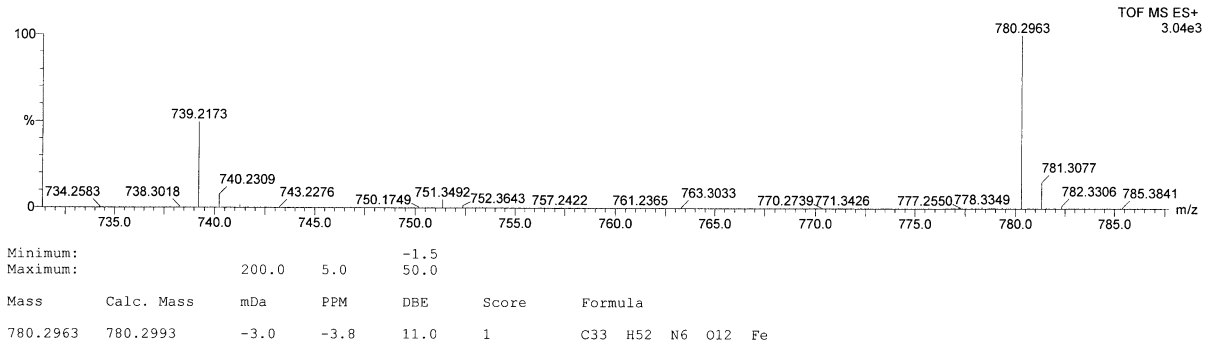

Fig. 4 The high-resolution ESI-MS spectra of the purified siderophore

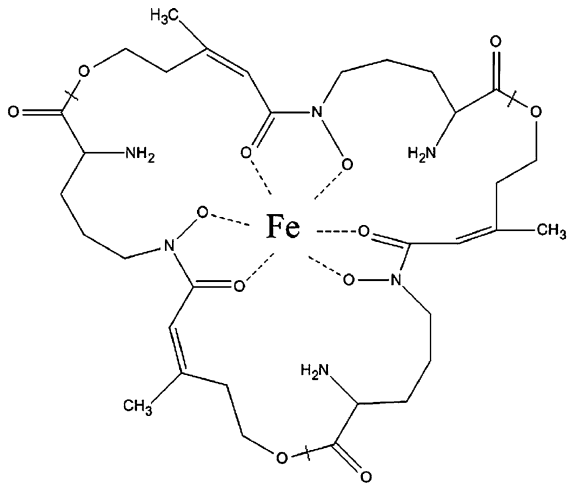

Fig. 5 The chemical structure of the purified siderophore. (†: ester bonds)

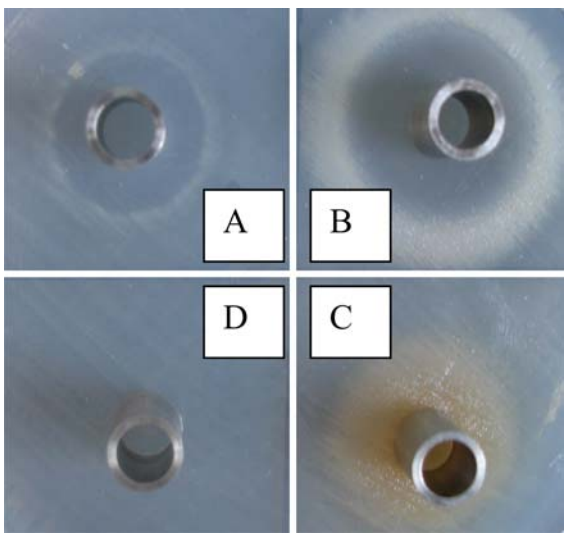

Fig. 6 Antibacterial activity of the crude and purified siderophore. **a** The crude siderophore. **b** The purified siderophore without Fe^{3+}. **c** The purified siderophore plus Fe^{3+}. **d** The purified siderophore chelated by Fe^{3+}

whereas Fe^{3+} represses siderophore production by the marine-derived yeast strain. In order to know if the stimulation and repression happen at transcriptional level, the amount of mRNAs encoding the hydroxylase in the cells grown in the medium (control), in the medium plus 10.0 mM of ornithine and in the medium supplemented with 2.0 μM of Fe^{3+}, were determined, respectively. The results in Fig. 7 indicated that added iron in the medium repressed expression of the hydroxylase gene in the yeast cells (Lane 6 in Fig. 7) while expression of the hydroxylase gene in the yeast cells grown in the medium plus ornithine was enhanced (Lane 4 in Fig. 7) compared to that in the yeast cells grown in the medium supplemented without anything (Lane 5 in Fig. 7). This means that iron has negatively affects on siderophore production and ornithine has stimulatory effects on siderophore production by the marine-derived yeast strain HN6.2 at the transcriptional level. It also can be observed clearly from the results in Fig. 7 that the amount of 18S rRNA was kept almost the same in the yeast cells grown under the different conditions (Lanes 1, 2 and 3 in Fig. 7).

Discussion

After purification by the reversed-phase HPLC and analysis by ESI-MS (Figs. 1, 2, 3, 4), we found that the siderophore produced by *A. pullulans* HN6.2 isolated from the sea salterns was fusigen which is one kind of the hydroxamate siderophores produced by fungi. This is the first report that the marine derived yeast can

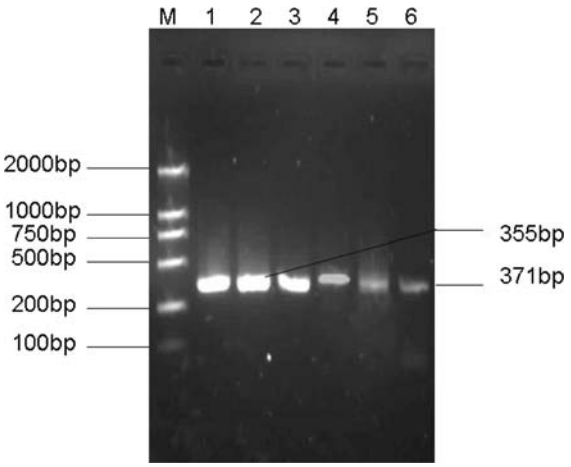

Fig. 7 The changes of the amount of mRNAs encoding the hydroxylase in the yeast cells grown in different media. *Lane 1*: The amount of 18S rRNA in the yeast cells grown in the production medium plus ornithine. *Lane 2*: The amount of 18S rRNA in the yeast cells grown in the production medium (control). *Lane 3*: The amount of 18S rRNA in the yeast cells grown in the production medium supplemented with iron. *Lane 4*: The amount of mRNA encoding the hydroxylase in the yeast cells grown in the production medium plus ornithine. 5: The amount of mRNA encoding the hydroxylase in the yeast cells grown in the production medium (control). *Lane 6*: The amount of mRNA encoding the hydroxylase in the yeast cells grown in the production medium supplemented with iron. M: DNA markers (the DNA bands from bottom to top are 0.1, 0.2, 0.5, 0.75, 1.0 and 2.0 kb)

produce this kind of siderophore. Fusigen, also called fusarinine C, has been reported earlier to be a typical hydroxamate siderophore of *Fusarium* species (Diekmann and Zahner 1967; Sayer and Emery 1968) and an endophyte which was isolated from the calcicolous ericaceous plant *Rhodothamnus chamaecistus* (L.) Rchb. growing on nearly neutral calcareous soil (Rendzina, pH 6.5) (Haselwandter et al. 1992). However, the endophyte also can produce ferricrocin, ferrichrome and monoacetylfusigen B in addition to fusigen (Haselwandter et al. 1992). In the proposed biosynthetic pathway for fungal siderophore biosynthesis, the first enzyme in the pathway is L-ornithine N^5-oxygenase (also termed ornithine N^5-hydroxylase) which catalyzes the N-hydroxylation of L-ornithine. The hydroxamate group is formed next with the attachment of an acyl group from acyl CoA derivatives by N^5-transacylases. The further reactions of siderophore biosynthesis are catalyzed by non-ribosomal peptide synthetases which are responsible for assembling siderophores by activating the precursors and incorporating these into a peptide, forming either peptide or ester bonds between the hydroxamate groups (Haas 2003; Johnson 2008). However, the chemical structure of fusigen obtained in this study indicates that the attachment of an acyl group from acyl CoA derivatives by N^5-transacylases does not happen in this compound (Fig. 5). Therefore, it is easy to investigate expression of the genes responsible for the fusigen biosynthesis in the marine-derived yeast under different environmental conditions.

The results in Fig. 6 demonstrate that only the siderophore that is not chelated by Fe^{3+} could inhibit the cell growth of the bacterial pathogen. Therefore, we still think that the siderophore produced by the yeast strain HN6.2 had much higher affinity for iron than that produced by the pathogenic bacterium used in this study, leading to the strong growth inhibition of the bacterium by depriving the bacterium of iron as *V. anguillarum* also can produce its own siderophores, anguibactin (Actis et al. 1986; Jalal et al. 1989; Hossain et al. 1998; Soengas et al. 2006). This structure shows a dimeric complex in which the anguibactin is a tetradentate ligand while fusigen (Fig. 5) is a hexadentate ligand. Therefore, it can be expected for fusigen to have a higher complexation constant than anguibactin and thus the fusigen can prevent the ferric anguibactin to be formed, and thus preventing growth of *V. anguillarum*. The marine bacterium *V. anguillarum* is responsible for vibriosis, a systemic disease of fish characterized by hemorrhagic septicemia. Outbreaks of the vibriosis world-wide result in high mortality rates of infected fish (Zhang et al. 2006). Therefore, it is economically important how to control the growth of this pathogenic bacterium in marine environments. As the siderophore produced by the marine-derived *A. pullulans* HN6.2 is very stable in the environments (Wang et al. 2009), it may have many advantages over the antimicrobial peptide (Li et al. 2007) which is temperature sensitive and easily attacked by proteinase in the environments when the siderophore is applied to biocontrol of the pathogenic bacterium in marine environments.

The expression of the gene encoding L-ornithine N^5-oxygenase can be repressed by Fe^{3+} available in the medium and enhanced by ornithine available in the medium at the transcriptional level (Fig. 7). Many results also show that transcriptional repression by Urbs1 of *sid1* gene encoding the ornithine N^5-oxygenase in *Ustilago maydis* in the presence of

Fe^{3+} requires interaction via zinc fingers with two closely spaced GATA-motifs located in the promoter region of *sid1* gene (Johnson 2008). Our results were in agreement with those from An et al. (1997), Haas et al. (1999) and Zhou et al. (1998). So far, it has been found that Aft1 in *Saccharomyces cerevisiaeis* is the major activator for the transcription of genes involved in reductive iron uptake (Johnson 2008). However, it is still completely unknown if such activator for the transcription of genes involved in the siderophore biosynthesis also exists in *A. pullulans* HN6.2 when it is grown in the medium containing L-ornithine. This is being investigated in this laboratory.

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