Isolation and Transcriptional Regulation of the Fructose 1,6-Bisphosphate Aldolase Gene *FBA1* in the Psychrophilic Yeast *Cystofilobasidium capitatum*

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In this study, we report the primary structure of fructose 1,6-bisphosphate aldolase encoded by *Cystofilobasidium capitatum FBA1* (*CcFBA1*) and the regulation of its gene expression. *CcFBA1* consists of a 1,080 bp ORF corresponding to a protein of 360 amino acid residues, and the calculated molecular weight of CcFba1p is 39,626 Da. The CcFba1p has both aldolase class-II signatures 1 and 2. *CcFBA1* is expressed at the same level during growth on several different carbon sources, although expression level may be slightly higher on glucose. Transcription of *CcFBA1* is not influenced by temperature or existence of oxygen, although it was reduced during the stationary phase. We show that CcFba1p is a constitutive enzyme in *C. capitatum*, and suggest that *FBA1* could be used as a marker for the detection of pectinolytic psychrophilic yeasts using the PCR method.

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Psychrophiles are microorganism which exhibit optimal growth at 15°C or lower. As psychrophiles are able to grow at low temperatures they occasionally cause spoilage of foodstuffs during preservation. Therefore, contamination of foodstuffs by psychrophiles must be avoided, and rapid methods to detect psychrophiles in foodstuffs are required.

Putrefaction of foods is caused by metabolism of some microorganisms, thus much study regarding food spoilage generally estimates the cell number of microorganisms in foods. Nowadays, to detect and estimate the cell number of living microorganisms contaminating foods, expression of essential genes can be observed and cell numbers estimated by various methods including lumino-assay, measurement by CO₂, DNA chip and quantitative PCR. Therefore, it is useful to identify new markers to detect psychrophiles in foodstuffs by the PCR method.

Psychrophilic cells synthesize a set of proteins

which may provide a protective mechanism and alter metabolism in order to adapt in cold environments. In *Saccharomyces cerevisiae*, the mesophilic yeast, low temperature responsive (*LOT*) genes have been extensively studied which are induced in response to cold to provide not only stress responses but also developmental and physiological changes¹⁾. In *S. cerevisiae*, *FBA1* encoding fructose 1,6-bisphosphate aldolase (FBA: EC 4.1.2.13) has been reported to be one of the *LOT* genes induced by cold stress¹⁾. FBA is a glycolytic enzyme that catalyzes reversible aldol cleavage or condensation of fructose 1,6-bisphosphate into dihydroxyacetone-phosphate and glyceraldehyde 3-phosphate. The enzyme fulfills an amphibolic function as it is involved in both catabolic (glycolysis) as well as anabolic pathways (gluconeogenesis and Calvin cycle). Therefore, we considered FBA to be a potential target to analyze the cold response of psychrophiles and to detect psychrophiles in the

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foodstuffs.

We selected the basidiomycetous yeast *Cystofilobasidium capitatum*, which is able to utilize pectin as a sole carbon source^{2),3)}, as a model for psychrophiles contaminating foodstuffs. Here, we report (i) cloning of the gene encoding the FBA from *C. capitatum*, and (ii) how FBA synthesis is regulated by various growth conditions, e.g., carbon sources, oxygen, growth phase and temperatures.

Materials and Methods

1. Strains, media and cultivation

C. capitatum strain PPY-1 was used as the wild-type strain. Complex Yeast-Peptide-Dextrose (YPD) and synthetic MI media⁴⁾ were used for the cultivation of strain PPY-1. The carbon source in the MI medium was one of the following; 1% (w/v) galacturonic acid, 1% (v/v) glycerol, 1% (w/v) glucose or 1% (w/v) pectin (DE 10% or 90%) (Sigma Chemicals, St. Louis, MO). The initial pH of the media was adjusted to 5.0. Cultivation (100 ml) was performed anaerobically or aerobically at 5°C, 10°C, 15°C, or 20°C with rotary shaking at 150 rpm in 500-ml Erlenmeyer flasks, and growth was followed by measuring the optical density at 660 nm.

Escherichia coli DH5 α , which was used for plasmid propagation, was grown at 37°C in 2xYT medium, when necessary being supplemented with ampicillin (50 μ g/ml).

2. Cloning and sequencing of the *CcFBA1* gene

Total RNA was extracted from the cells grown on pectin by the acid-guanidinium thiocyanate-phenol-chloroform method using ISOGEN (Nippon Gene Co., Ltd., Toyama, Japan), and reverse transcribed according to the instructions of the 3'-Full RACE core set (Takara Shuzo Co., Ltd., Kyoto, Japan).

The cDNA encoding the FBA was cloned by chance when we were cloning the cDNA encoding polygalacturonase from *C. capitatum* by 3'-race PCR using F-1 primer; 5'-gTNgCNgCNgCNAARAARTgYAC-3', and Oligo dT adaptor primer (Takara Shuzo Co., Ltd.). The amplified ca 1.3-kbp fragment was cloned into the pT7blue T-vector (Novagen Inc., Madison, WI, USA). The DNA fragment was sequenced by the dideoxy cycle sequencing method with a PRISM DyeDeoxy Terminator Cycle Sequencing kit and a DNA sequencer model 3130 (Applied BioSystems, Foster, CA, USA). The nucleotide sequence of *CcFBA1* has been submitted

to DDBJ/EMBL/GenBank databases under the accession no. AB 244669.

The CLUSTAL X version 1.8 program was used for multiple alignment of sequences, construction of neighbor-joining phylogenetic tree⁵⁾, and 1,000 times bootstrap analysis for evaluation of phylogenetic tree topology⁶⁾.

3. Northern analysis

Total RNA was extracted from the cells by the acid-guanidinium thiocyanate-phenol-chloroform method using ISOGEN (Nippon Gene Co., Ltd.). Briefly, 20 μ g of an RNA sample was electrophoresed on a 1.0% agarose gel containing 20 mM MOPS buffer, 1 mM EDTA, and 2.2 M formaldehyde.

After electrophoresis, capillary transfer to a nylon membrane (Hybond-N+; Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK) in 20 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) was performed. The DNA probe including of the entire coding region of *CcFBA1* was labeled with an AlkPhos DIRECT kit (Amersham Pharmacia Biotech UK Ltd.).

Results and Discussion

1. Primary structure of *C. capitatum FBA1*

We cloned the complete *C. capitatum FBA1* cDNA gene. The *CcFBA1* consists of a 1,080 bp ORF corresponding to a protein of 360 amino acid residues and has 180 bases in the 5'-untranslated region, as shown in Fig. 1. The calculated molecular weight of CcFba1p is ca. 39, 626 Da, and its deduced amino acid sequence shows high similarity to those of the Fba1p from *Filobasidiella neoformans* var. *neoformans* JEC21 (72% identity), the *Ustilago maydis* Fba1p (66% identity), and the *Neurospora crassa* Fba1p (64% identity). The CcFba1p contains the aldolase class-II signature 1, which is located in the first half of the sequence and contains two histidine residues that have been shown to be involved in binding a zinc ion⁷⁾. Furthermore, CcFba1p contains the aldolase class-II signature 2, which is located in the C-terminal section and contains clustered acidic and glycine residues (Fig. 1). Until now, two distinct classes of FBAs occur in nature, which differ in their enzymatic mechanisms⁸⁾. Class-II aldolases are mainly found in prokaryotes and fungi, and they are homodimeric enzymes which require a divalent metal ion, generally zinc, for their activity. These facts show that CcFba1p, gene product of *CcFBA1*, is a class II aldolase, and is able

Fig. 1 Nucleotide and deduced amino acid sequences of the *C. capitatum* *FBA1* gene

The gray and white boxes indicate the aldolase class-II signature 1 and signature 2, respectively.

to function as an FBA. In short, *CcFBA1* encodes functional FBA in *C. capitatum*.

Moreover, we constructed a phylogenetic tree for FBAs of class II based on CcFba1p and other fungal FBAs. Fungal FBAs of class II compose two distinct subgroups, one primarily is basidiomycetes and the other is ascomycetes (Fig. 2). This phylogenetic pattern agreed with that using 28 S rDNA (data not shown). This finding showed that CcFba1p evolved in basidiomycetes, and its evolution has been hardly affected by cold environments.

2. Regulation of the *CcFBA1* gene in various growth conditions.

To obtain a regulatory insight into the expression of *FBAI* by several carbon sources in *C. capitatum*, we performed Northern analysis using a specific probe for the *CcFBAI*.

At first, *CcFBAI* expression in *C. capitatum* grown on several carbon sources was monitored. As shown in Fig. 3, *CcFBAI* was expressed during growth using glucose, glycerol, galacturonic acid and pectin, and generally expression levels were similar for each carbon source, although expression level on glucose was slightly higher than the others. Transcription of *FBAI* genes in *S. cerevisiae* and *K.*

Fig. 2 Phylogenic relationships of class II FBAs from several fungal strains

The branching pattern was generated by the neighbor-joining method. The bar indicates 0.05 nucleotide substitutions per site. FBAs from: SpFba1p, *Schizosaccharomyces pombe* (AL109731); UmFba1 p, *U. maydis* (AX710044); FnFba1 p, *F. neoformans* var. *neoformans* (XM568771); CcFba1 p, *C. capitatum* (AB244669); AoFbaAp, *Aspergillus oryzae* (AB032272); PbFbaAp, *Paracoccidioides brasiliensis* (AY057387); NcFbaAp, *N. crassa* (L42380); YlFba1p, *Yarrowia lipolytica* (CR382131); ScFba1 p, *S. cerevisiae* (X15003); and AgFba1p, *Ashbya gossypii* (AE016815).

Fig. 3 Regulation of the *CcFBA1* expression in *C. capitatum*

Total RNA (20 μ g) was loaded on each lane and probed with the *CcFBA1*.

(A) Effect of carbon sources. Growth conditions of each lane are : lane 1, 1 % (w/w) galacturonic acid ; lane 2, 1 % (v/w) glycerol ; lane 3, 1 % (w/w) glucose (anaerobic) ; lane 4, 1 % (w/w) glucose (aerobic) ; lane 5, 1 % (w/w) pectin (DE 10%) ; and lane 6, 1 % (w/w) pectin (DE 94%).

(B) Effect of temperature. Growth conditions of each lane are : lane 1, 5 °C ; lane 2, 10 °C ; lane 3, 15 °C ; and lane 4, 20 °C on pectin (DE 90%) medium.

lactis was not greatly influenced by the carbon source present in the media, *e.g.*, glucose, glycerol and lactate^{(9), (10), (11)}. It appears that *CcFBAI* is not regulated by catabolite repression. Oxygen did not influence the expression of *CcFBAI* (Fig.3), although the expression of *KIFBAI* is regulated by oxygen concentration. Expression of *KIFBAI* mRNA

decreased during the shift from hypoxic to aerobic conditions and increased when these conditions were reversed¹¹⁾.

ScFBAI has been reported to be a *LOT* (low temperature responsive) gene which is induced by cold stress¹⁾. Since *C. capitatum* belongs to the psychrophilic yeast, we investigated whether expression of *CcFBAI* was regulated by growth temperature or not. However, *CcFBAI* expressed at the same level at several growth temperatures (Fig. 3 B).

CcFBAI expressed strongly during lag phase, and its expression was reduced during stationary phase, although the gene is constitutively expressed (Fig. 4). These findings show *CcFBAI* is mainly expressed during cell division. Moreover, the gene is essential like the rRNA genes, but unlike rRNA gene expression patterns *CcFBAI* expression was reduced during the stationary phase. Therefore, expression of *CcFBAI* may be a marker for cell duplication, and the gene is potentially useful for the detection of microorganisms in putrid foods.

Fig. 4 Growth curve and expression ratio of the *CcFBAI* in *C. capitatum*

(A) Growth curve of *C. capitatum* on pectin (●), and relative density of band corresponding with the *CcFBAI* in Northern analysis.

(B) Northern analysis.

3. Conclusion

In this study, we revealed the primary structure of FBA encoded by *C. capitatum FBAI* and the regulation of its gene expression. The CcFba1p contains the aldolase class-II signature 1 and signature 2²⁾. *CcFBAI* is expressed constitutively and is not regulated by catabolite repression. These results show that *CcFBAI* encodes functional FBA in *C. capitatum* and CcFba1p has the ability to function as an FBA.

Expression of *CcFBAI* is not influenced by growth temperature. In *S. cerevisiae*, *ScFBAI* has been reported to be one of the *LOT* genes induced by cold stress¹⁾. We think that this difference between the regulation of *FBAIs* from *S. cerevisiae* and *C. capitatum* is related to the difference of growth features between these strains, i.e., *S. cerevisiae* is mesophilic, whereas *C. capitatum* is psychrophilic. It appears that *C. capitatum* has developed regulatory mechanisms of *CcFBAI* expression in order to adapt to cold habitats. Based on these facts, we propose that psychrophiles have evolved different gene regulatory systems besides cold-active enzymes to survive in low-temperature environments.

CcFBAI is expressed constitutively. Expression of *CcFBAI* is not influenced by lag and log phases and by presence of oxygen, although it was reduced during the stationary phase. We conclude that FBA could be a good target for the detection and quantification of psychrophiles in foodstuffs by the PCR method.

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低温性酵母 *Cystofilobasidium capitatum* 由来 アルドラーゼの一次構造とその発現制御

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本研究では、低温性ペクチン資化性酵母 *Cystofilobasidium capitatum* 由来アルドラーゼの一次構造とその発現制御について解析を行った。*C. capitatum* のアルドラーゼをコードする遺伝子 *FBA1* は360アミノ酸残基をコードしうるORFからなり、その遺伝子産物CcFba1pはアルドラーゼに特有の保存配列を保持しており、推定分子量は39,626Daであった。一方、*FBA1* はグルコースにより若干の高発現がみられるものの、炭素源や温度、酸素濃度にはほとんど影響されなかった。さらに、*FBA1* は定常期において発現の低下がみられるものの、誘導期および対数増殖期においては安定した発現が観察された。これらの結果から、*FBA1* は *C. capitatum* において構成的に発現されるハウスキーピング遺伝子の一つであり、本遺伝子が食品中におけるこれら低温性酵母の検出の指標に用いることができる可能性を示した。

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