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Yeast Sequencing Report

Isolation and sequence analysis of the gene encoding triose phosphate isomerase from *Zygosaccharomyces bailii*

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Abstract

The *ZbTPI*1 gene encoding triose phosphate isomerase (TIM) was cloned from a *Zygosaccharomyces bailii* genomic library by complementation of the *Saccharomyces cerevisiae tpi1* mutant strain. The nucleotide sequence of a 1.5 kb fragment showed an open reading frame (ORF) of 746 bp, encoding a protein of 248 amino acid residues. The deduced amino acid sequence shares a high degree of homology with TIMs from other yeast species, including some highly conserved regions. The analysis of the promoter sequence of the *ZbTPI1* revealed the presence of putative motifs known to have regulatory functions in *S. cerevisiae*. The GenBank Accession No. of *ZbTPI1* is AF325852.

Keywords: **glycolysis; triose phosphate isomerase; spoilage yeast; *Zygosaccharomyces bailii***

Introduction

S. cerevisiae, well known for its use in the production of food and beverages, is a yeast of ongoing applications in a number of different processes within the pharmaceutical industry. Considering the biodiversity existing among yeasts, recent attention has been focused on other yeast genera that display peculiar traits more suitable for industrial applications. In this context, *Zygosaccharomyces* spp., closely related to *Saccharomyces*, are of particular interest due to their ability to survive under various stress and environmental conditions. *Z. bailii* is known for its ability to grow on mixtures of sugars in the presence of high concentrations of acetic acid and low pH values (Sousa *et al*., 1996, 1998). In contrast to *S. cerevisiae*, which always prefers glucose as carbon source, *Z. bailii* exhibits the phenomenon of so-called fructophily. In fact, when glucose and fructose are both available in the medium, *Z. bailii* incorporates fructose more rapidly (Sousa-Dias *et al*., 1996). However, sugar metabolism in this microorganism has not so far been extensively studied. In yeasts, as in many organisms, the primary metabolic flux is sugar metabolism through the glycolytic pathway. In *S. cerevisiae*, the glycolytic enzymes constitute as much as 30–60% of the soluble proteins (Fraenkel, 1982). These values are determined from the high level of expression of the individual genes; the promoters of these genes have been used in *S. cerevisiae* for high expression of heterologous proteins. Moreover, the glycolytic enzyme triose phosphate isomerase (TIM E.C.5.3.1.1) plays a relevant role in sugar metabolism. It catalyses the interconversion between dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP), which fall into important metabolic branch points between glycolysis, gluconeogenesis, the pentose pathway, glycerol production/utilization and the methylglyoxal pathway (Gancedo and Serrano, 1989; Cooper, 1984). In *S. cerevisiae* the lack of TIM activity causes an accumulation of DHAP, which leads to a redirection of the NADH reoxidation (Compagno *et al*., 1996). In such a mutant, a high synthesis of glycerol was observed (Compagno *et al*., 1996). In

Kluyveromyces lactis, a similar effect has been recently reported (Compagno *et al.*, 1999). All the peculiar traits of *Z. bailii* indicate this yeast as a promising candidate for glycerol synthesis and production of heterologous proteins (Brambilla *et al.*, 2000). In this connection, it was attempted to isolate the gene encoding TIM from *Z. bailii* to facilitate the study of sugar metabolism and glycerol production. Furthermore, the promoter of this gene could be very useful during the construction of vectors leading to efficient heterologous gene expressions in *Z. bailii*.

Materials and methods

Yeast strains and media

Z. bailii ISA 1307, originally isolated as a spoilage agent from a continuous production plant of sparkling wine (Wium *et al.*, 1990), was obtained from the Culture Collection of the Instituto Superior de Agronomia (Lisbon, Portugal). *S. cerevisiae* W303Δ*TPI1* (MATα, *tpi1::kan*^{r}, *ade2-1*, *can1-100*, *ura3-1*, *leu2-3*,*112*, *trp1-1*, *his3-11*,*15*; Compagno *et al.*, 1996) displays the TIM-deficient phenotype and was used for complementation assays. Cells of *S. cerevisiae* W303Δ*TPI1* were grown in rich media (2% w/v peptone, 1% w/v yeast extract) or minimal media (6.7 g/l Yeast Nitrogen Base, adenine 100 mg/l, uracil 50 mg/l, leucine 50 mg/l, tryptophan 50 mg/l, histidine 50 mg/l) containing both glucose (0.1%, w/v) and ethanol (1%, v/v) as carbon and energy sources. *Z. bailii* was cultivated in rich media (2% w/v peptone, 1% w/v yeast extract) or minimal media (6.7 g/l Yeast Nitrogen Base) containing glucose (2%, w/v) as carbon and energy source.

Isolation and sequencing of the *ZbTPI1* gene

A genomic library from *Z. bailii* ISA 1307 constructed in the shuttle vector pRS316 for *E. coli* and *S. cerevisiae* (centromeric; selectable marker: *URA3*) was used (Rodrigues *et al.*, 1999). The strain of *S. cerevisiae* Δ*TPI1* was transformed, with this genomic library, by the lithium acetate procedure (Geitz *et al.*, 1995). Ura$^+$ transformants were replicated on minimum plates containing glucose (2%, w/v). Plasmid-dependent growth of the transformants on this medium was verified by plasmid rescue (Hoffman *et al.*, 1987). Plasmid DNAs extracted from the *E. coli* were mapped. The restriction fragment *Hin*dIII–*Xba*I from a 1.5 kb clone was subcloned into the vector pGEM–7Zf (Promega) and sequenced on both strands by BioStrands S.r.l. (AREA Science Park, Padriciano 99 34012 Trieste, Italy).

Enzyme assay

Cell extracts were prepared essentially as previously described (Compagno *et al.*, 1999). Protein content of cell extracts was determined with Bio-Rad kit #500-002, using bovine serum albumin as a standard. The specific activity of the triose phosphate isomerase was determined on cell extracts, in triethanolamine (50 mM)–$MgCl_2$ (10 mM) buffer, pH 7.4, 0.3 mM NADH, and 1 U/ml glycerol-3-phosphate dehydrogenase (GDH) (Boehringer). The reaction was started by addition of 0.4 mM glyceraldehyde 3-phosphate (3-PGA) (Sigma).

Results and discussion

Cloning of the *Z. bailii* gene complementing a *S. cerevisiae tpi1* mutant

A *S. cerevisiae* strain lacking triose phosphate isomerase activity is unable to grow on glucose media (Compagno *et al.*, 1996). We used this defective phenotype to search for the *Z. bailii* gene coding for TIM by functional complementation. A genomic DNA library of *Z. bailii* was used to transform the strain W303 Δ*TPI*1 of *S. cerevisiae* and Ura$^+$ transformants were selected on minimal medium plates containing both glucose (0.1%, w/v) and ethanol (1%, v/v) as carbon and energy sources (i.e. permissive condition for Δ*TPI*1 cells). 3500 Ura$^+$ transformants were transferred by replica plating to plates containing glucose (2%, w/v) as carbon and energy source. From this selection, two clones were obtained in which the growth on

Table 1. TIM specific activities (Units per mg of total protein extract) from cell free extracts of *Z. bailii* ISA 1307, *S. cerevisiae* W303 1A, *S. cerevisiae* W303 1A ΔTPI1 and the TIM-deficient strain complemented with *ZbTPI1*

Yeast strain	TIM activity (unit/mg protein)
Z. bailii ISA1307	11.4
S. cerevisiae W303	14.1
W303 Δ*TPI1*	0.0
W303 Δ*TPI1* complemented	15.5

glucose was restored. Enzyme assays showed that, in both transformants growing in minimal medium, TIM activity was restored (Table 1). Plasmids were isolated from TIM^+ yeast colonies, amplified in *E. coli* and analysed. The restriction maps revealed two regions of about 3.5 kb and 1.5 kb, both able to complement the Δ*TPI*1 phenotype. The shortest one was subcloned and selected for further studies.

Sequence analysis of *ZbTPI1* gene

The insert on the selected plasmid was sequenced on both strands. The nucleotide sequence of the gene thus obtained and the deduced amino acid sequence of the encoding polypeptide are shown in Figure 1. An open reading frame (ORF) of 746 bp was identified, encoding a protein of 248 amino acid residues (Figure 1).

```
-528  CCAAGCTTGATATCGAATTCCTGCAGCCCGGGGGATCGTATTGCTTCCATTCTTCTTTTG  -469
-468  TTATTCGGCGCGATTCGAATTCATGACATCTTTTAACCGTCCGCACTACATTACTGGCTC  -409
-408  AAGAAAGGATTGATAAATACTACCAAGGAACACGTGTATCCATTTGATACTGTGCTGGTT  -349
-348  ACAAGACACATGCTTTACAAGCACACTTCTATCTCTCTCGACTGAGGCGAAACGTCGAGT  -289
-288  GGTTTGATATCAAATGCATGCGTGATATGCACCATTATTTTTCCCTTTTACTTCCGTCAC  -229
-228  GCCGGGGCTCCACTTTTTTGGGTTCCACTTTTCTTACGACCCTCGACATCCACTAAACGA  -169
-168  ACAGGAAGTCAAAGAACCCCTCGAGTCACACGGTGCGTATGCGCTGTTAACATATATAAA  -109
-108  GGTCACCTTTCCCTGCTCAAAAGAGTCTTAGCAGGCTGTTAACTTCACTCTCTATCGATC  -49
 -48  CATAGAATCTAACTAACAAGAGACTACATCGGTATAACAAATAACAAAATGGCTAGAACT  12
                                                      M  A  R  T
  13  TTTTTCGTCGGCGGTAACTTCAAACTAAACGGTACCAAGTCCAGCATCAAGGAGATTGTT  72
        F  F  V  G  G  N  F  K  L  N  G  T  K  S  S  I  K  E  I  V
  73  GAGAGACTAAACAACGCTAAGTTGGACCCTAAGGTCGAGGTGGTGTTGTGTCCTCCAGCC  132
        E  R  L  N  N  A  K  L  D  P  K  V  E  V  V  L  C  P  P  A
 133  CCATACTTGGACTACACCGTCTCCCTTGTCAAGAAGTCTCAGGTTTCCGTCGGTGCCCAG  192
        P  Y  L  D  Y  T  V  S  L  V  K  K  S  Q  V  S  V  G  A  Q
 193  AACGCTTACTTGAAGGCTTCCGGTGCCTTCACTGGTGAGAACTCTGTTGACCAGATCAAG  252
        N  A  Y  L  K  A  S  G  A  F  T  G  E  N  S  V  D  Q  I  K
 253  GATGTTGGTGCTAAGTGGGTCATTCTAGGTCACTCCGAGAGAAGACAGTATTTCAGGGAA  312
        D  V  G  A  K  W  V  I  L  G  H  S  E  R  R  Q  Y  F  R  E
 313  GACGACCAATTGATCGCCGAGAAGACTGCCTTTGCTCTTTCTCAGGGTGTTGGTGTCATC  372
        D  D  Q  L  I  A  E  K  T  A  F  A  L  S  Q  G  V  G  V  I
 373  TTGTGTATTGGTGAGACTCTTGACCAAAAGAAGGCTGGTACCACTCTTCAGGTTGTCGAG  432
        L  C  I  G  E  T  L  D  Q  K  K  A  G  T  T  L  Q  V  V  E
 433  AGACAACTACAGGCTGTCATTGACAAGGTCAAGGACTGGTCCAACGTTGTTATTGCTTAC  492
        R  Q  L  Q  A  V  I  D  K  V  K  D  W  S  N  V  V  I  A  Y
 493  GAGCCTGTGTGGGCTATTGGTACTGGTCTAGCTGCTACCCCAGAGGATGCTCAGGAAATC  552
        E  P  V  W  A  I  G  T  G  L  A  A  T  P  E  D  A  Q  E  I
 553  CACCACTCCATCAGAGAATTCTTGGCTAAGAAGCTGGGTGAGAAGACCGCTCAGGAGACT  612
        H  H  S  I  R  E  F  L  A  K  K  L  G  E  K  T  A  Q  E  T
 613  AGAATCCTATACGGTGGTAGTGCCAACGGTAAGAACGCTGTCACTTTCAAGGACAAGCCA  672
        R  I  L  Y  G  G  S  A  N  G  K  N  A  V  T  F  K  D  K  P
 673  GACGTTGACGGTTTCTTGGTTGGTGGTGCCTCTTTGAAGCCAGAGTTCGTTGACATCATC  732
        D  V  D  G  F  L  V  G  G  A  S  L  K  P  E  F  V  D  I  I
 733  AACTCTAGATCTTGAGGAAAGCATGTGCATTGACGGCAAGTACTAGAACGATAGGTTACG  792
        N  S  R  S  *
 793  AAATAGAAGCAATATATATAATGATATAAAGATCTGTCACGACAACCCCATTCATTTCGA  852
 853  TTTTCTGCCAAAGCTAGGTGAGGATACTGTAGCAGCTGGATCTGGGGTACCTTTGTTGTA  912
 913  AGTCAGCCCATTGAACAACAAACAGGTCGGCCAGGTAGTAAAGGATTTTACCTGTGAGAG  972
 973  AAAGAGATCCACTAGTTCTAGAGGAGCATGCGACGTCG   1010
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Figure 1. Nucleotide sequence of *Z. bailii TPI*1 gene. The predicted amino acid residues of the ORF are shown below the nucleotide sequence (see also text)

In the putative promoter region of the gene (Figure 1), a TATAAA element was present at position −114. A search for the presence of regulatory elements revealed a putative STRE motif (Moskvina *et al.*, 1998) at −152. No consensus sequence for the GRF1/RAP1 binding site was found; however, such a sequence may be located upstream of the fragment sequenced. Moreover, two CTTCC blocks, that are present in *S. cerevisiae* TPI1 promoter and are known to be implicated in high level of expression of glycolytic genes in *S. cerevisiae* (Chambers *et al.*, 1989; Scott *et al.*, 1990), were found at positions −238 and −485.

The deduced protein sequence of the *Z. bailii* TIM was compared to the sequence of other TIMs reported in GenBank, using the BLAST programme. The highest level of identity was observed for the TIM of *S. cerevisiae* (83%) and *K. lactis* (81%); the TIM of *Schizosaccharomyces pombe* showed a lower identity (52%) (Figure 2). The sequence alignment of the TIM from *Z. bailii* and TIMs from other organisms revealed that the most conserved regions contain the known structural and functional domains of the enzyme (Lolis *et al.*, 1990; Wierenga *et al.*, 1992). Since the inserted DNA fragment complemented the TIM$^-$ phenotype in *S. cerevisiae* and showed a high degree of identity with those encoding TIM of other organisms, the gene isolated was therefore named as *ZbTPI1*.

```
                1                                                    50
TPIS-Zb         RTFFVGGNFK LNGTKSSIKE IVERLNNAKL DPK...VEVV LCPPAPYLDY
TPIS-Sc         RTFFVGGNFK LNGSKQSIKE I.VERLNTAS IPEN..VEVV ICPPATYLD.
TPIS-Kl         .......... .......... .......... .......... ..........
TPIS-Szp        RKFFVGGNFK MNGSLESMKT I.IEGLNTTK LNVGD.VETV IFPQNMYL..
TPIS-Ec         RHPLVMGNWK LNGSRHMVHE LVSNLRKELA GVA.GCAVAI .APPEMYIDM
TPIS-Dm         RKFCVGGNWK MNGDQKSIAE I.AKTLSSAA LDPN..TEVV IGCPAIYLM.
TPIS-Hs         RKFFVGGNWK MNGRKQSLGE L.IGTLNAAK VPAD..TEVV CAPPTAYID.
TPIS-Zm         RKFFVGGNWK CNGTTDQVEK I.VKTLNEGQ VPPSDVVEVV VSPPYVFLP.
Consensus       r.....gNwK .n........ .......... ......v... ..pp...l..

                51                                                   100
TPIS-Zb         TVSLVKKS.. ......QVSV GAQNAYLKAS .GAFTGENSV DQIKDVGAKW
TPIS-Sc         ...YSVSLVK ....KPQVTV GAQNAYLKAS .GAFTGENSV DQIKDVGAKW
TPIS-Kl         .......... .......... .......... .......... ..........
TPIS-Szp        ...ITTRQQV ....KKDIGV GAQNVFDKKN .GAYTGENSA QSLIDAAITY
TPIS-Ec         AKREAEG... .....SHIML GAQNVDLNLS .GAFTGETSA AMLKDIGAQY
TPIS-Dm         ...Y.ARNLL ....PCELGL AGQNAYKVAK .GAFTGEISP AMLKDIGADW
TPIS-Hs         ...F.ARQKL ....DPKIAV AAQNCYKVTN .GAFTGEISP GMIKDCGATW
TPIS-Zm         ...V.VKSQL ....RQEFHV AAQNCWVKKG .GAFTGEVSA EMLVNLGVPW
Consensus       .......... .......... .aQn...... .Ga.TGe.s. ..l.#.g...

                101                                                  150
TPIS-Zb         VILGHSERRQ YF...REDDQ LIAEKTAFAL SQGVGV.ILC IGETLDQKKA
TPIS-Sc         VILGHSERRS YF...HEDDK FIADKTKFAL GQGVGV.ILC IGETLEEKKA
TPIS-Kl         .......... .......... .......... ........LC IGETLEEKQQ
TPIS-Szp        TLTGHSERRT IF...KESDE FVADKTKFAL EQGLTV.VAC IGETLADREA
TPIS-Ec         IIIGHSERRT YH...KESDE LIAKKFAVLK EQGLTP.VLC IGETEAENEA
TPIS-Dm         VILGHSERRA IF...GESDA LIAEKAEHAL AEGLKV.IAC IGETLEEREA
TPIS-Hs         VVLGHSERRH VF...GESDE LIGQKVAHAL AEGLGV.IAC IGEKLDEREA
TPIS-Zm         VILGHSERRA LL...GESNE FVGDKVAYAL SQGLKV.IAC VGETLEQREA
Consensus       vi.gHSErR. ......#..e ....k...al ..g......c .ge.l...ea

                151                                                  200
TPIS-Zb         GTTLQVVERQ LQAVIDKVKD W........S NVVIAYEPVW AIGTGLAATP
TPIS-Sc         GKTLDVVERQ LNAVLEEVK. ..D.W....T NVVVAYEPVW AIGTGLAATP
TPIS-Kl         NITLQVVQRQ LQAVLEKVQD W........T NVVVAYEPVW AIGTGLAATA
TPIS-Szp        NETITVVVRQ LNAIADKVQ. ..N.W....S KIVIAYEPVW AIGTGKTGTP
TPIS-Ec         GKTEEVCARQ IDAVLKTQGA ..AAF....E GAVIAYEPVW AIGTGKSATP
TPIS-Dm         GKTNEVVARQ MCAYAQKIK. ..D.W....K NVVVAYEPVW AIGTGKTATP
TPIS-Hs         GITEKVVFEQ TKVIADNVK. ..D.W....S KVVLAYEPVW AIGTGKTATP
TPIS-Zm         GSTMDVVAAQ TKAIAE.KIK ..D.W....S NVVVAYEPVW AIGTGKVATP
Consensus       g.t..v...q .......... .......... ..v.ayep.w aigtgk.a..
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Figure 2. *Continues on next page*

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          201                                                        250
TPIS-Zb   EDAQEIHHSI RREFLAKKLGE KTAQETRILY GGSANGKNAV TFKDKPDVDG
TPIS-Sc   EDAQDIHASI RKFLASKLGD KAASELRILY GGSANGSNAV TFKDKADVDG
TPIS-Kl   EDAQDIHHSI RREFLAEKLSR DVADSVRILY GGSANGKNAV TFKDKADVDG
TPIS-Szp  EEAQEVHAEI RKWATNKLGA SVAEGLRVIY GGSVTGGNCK EFLKFHDIDG
TPIS-Ec   AQAQAVHKFI RDHIAKV.DA NIAEQVIIQY GGSVNASNAA ELFAQPDIDG
TPIS-Dm   DQAQEVHASL RQWLSDNISK EVSASLRIQY GGSVTAANAK ELAKKPDIDG
TPIS-Hs   QQAQEVHEKL RGWLKSNVSD AVAQSTRIIY GGSVTGATCK ELASQPDVDG
TPIS-Zm   AQAQEVHASL RDWLKTNASP EVAESTRIIY GGSVTAANCK ELAAQPDVDG
Consensus ..a...h... r......... ..a....i.y ggsv...n.. .......idg

          251                    277
TPIS-Zb   FLVGGASLK. PEFVDIINSR .......
TPIS-Sc   FLVGGASLK. PEFVDIINSR .......
TPIS-Kl   FLVGGASLK. PEFVDIINSR .......
TPIS-Szp  FLVGGASLKP EFPTNIVN.. .......
TPIS-Ec   ALVGGASLKA DAFAVIVKAA EAAK...
TPIS-Dm   FLVGGASLK. PEFLDIINAR Q......
TPIS-Hs   FLVGGASLK. PEFVDIINAK Q......
TPIS-Zm   FLVGGASLK. PEFIDIINAA TVKS...
Consensus .lvggasl.. ..f..i.... .......
```

Figure 2. Amino acid sequence alignment of TIMs from different organisms obtained by using Prodom-search (**http://protein.toulouse.inra.fr/prodom/blast_form.html**). Zb, *Z. bailii* (Accession No. AF325852); Sc, *S. cerevisiae* (Accession No. J01366); Kl, *K. lactis* (Accession No. AJ012317); Szp, *Sz. pombe* (Accession No. M14432); Ec, *E. coli* (Accession No. X00617); Dm, *D. melanogaster* (Accession No. X57576); Hm, *Homo sapiens* (Accession No. M10036); Zm, *Zea mais* (Accession No. D00012). Consensus sequences are underlined in **bold**. #Any one of NDQEBZ

The specific activity of TIM was determined from cell-free extracts of *S. cerevisiae* and *Z. bailii* strains growing in minimal media (Table 1). Similar levels of enzyme specific activity were detected in the wild-type strains of *Z. bailii* and *S. cerevisiae* and in the *S. cerevisiae* Δ*TPI1* mutant complemented with *ZbTPI1*.

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