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Cloning, Expression and Identification of a New Trehalose Synthase Gene from *Thermobifida fusca* Genome

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Abstract A new open reading frame in *Thermobifida fusca* sequenced genome was identified to encode a new trehalose synthase, annotated as "glycosidase" in the GenBank database, by bioinformatics searching and experimental validation. The gene had a length of 1830 bp with about 65% GC content and encoded for a new trehalose synthase with 610 amino acids and deduced molecular weight of 66 kD. The high GC content seemed not to affect its good expression in *E. coli* BL21 in which the target protein could account for as high as 15% of the total cell proteins. The recombinant enzyme showed its optimal activities at 25 °C and pH 6.5 when it converted substrate maltose into trehalose. However it would divert a high proportion of its substrate into glucose when the temperature was increased to 37 °C, or when the enzyme concentration was high Its activity was not inhibited by 5 mM heavy metals such as Cu^{2+}, Mn^{2+}, and Zn^{2+} but affected by high concentration of glucose. Blasting against the database indicated that amino acid sequence of this protein had maximal 69% homology with the known trehalose synthases, and two highly conserved segments of the protein sequence were identified and their possible linkage with functions was discussed.

Key words trehalose synthase; *Thermobifida fusca*; open reading frame (ORF); gene expression

Trehalose is a disaccharide with two glucose molecules linked in an α,α-1,1-glycosidic linkage. The only reducing group in each of its glucose molecules has been used up for the formation of α,α-1,1-glycosidic linkage, therefore trehalose is a nonreducing disaccharide with high stability against the disruption caused by such factors as temperature and extreme pH of environment [1]. It has been well established that many organisms will cope with external stress conditions by increasing the level of cytosolic trehalose [2]. This phenomenon suggests that trehalose may somehow have a protecting function for the cell components. Investigation on the cell membranes of anhydrobiotic organisms [3] has unraveled that intracellular trehalose can indeed protect proteins and cellular membrane from inactivation or denaturation caused by a variety of stress conditions such as desiccation, dehydra-tion, heat, cold and even oxidation. One of most important characteristics of trehalose is that it not only protects biomolecules *in vivo* but also has the same protection effect *in vitro*. This rarely paralleled feature of disaccharides has opened a new field for its application in food industry [4] and pharmaceutical manufacturing practice [5].

For the microorganisms, there are at least three pathways for the biosynthesis of trehalose reported so far in the literature. The earliest known and most widely distributed pathway is catalyzed by the trehalose-6-phosphate synthase, which condenses UDP-glucose and glucose-6-phosphate to form trehalose-6-phosphate which further dephosphorylated to trehalose by trehalose-6-phosphate phosphatase [6]. This pathway universally exists from extremophiles to animals, and has recently been explored to overproduce trehalose by expressing the corresponding *E. coli* genes in *Corynebacterium glutamicum* [7]. The second pathway involves the rearrangement of internal glycosidic linkage between the molecules of glucose polymer such as maltooligosaccharides produced from the

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hydrolysis of starch using α-amylase [8,9]. The enzyme α-amylase, which is encoded by maltooligotrehalose synthase gene *tre*Y, can convert the α-(1-4) linkage of the terminal residue of the maltooligosaccharides into an α-(1-1) linkage. This pathway has been found to exist in many species, especially in archaebacteria such as *Sulfolobus acidocaldarius* [9], *Sulfolobus solfataricus* KM1 [10], *Sulfolobus ahibatae* [11,12], and some mesophilics such as *Brevibacterium helvolum* [13]. The third pathway is somehow similar to the second pathway in the sense of internal rearrangement of the glycosidic linkage between the molecules, but it can only use maltose as its substrate, and the enzyme responsible for this type of chemical bonding reshuffle [14] is trehalose synthase (maltose α-D-glucosyltransferase, EC.5.4.99.16) which is encoded by gene *tres*. This pathway has been found in *Pimelobacter sp*. R48 [15], *Thermus aquaticus* ATCC33923 [16] and *Thernmus caldophilus* GK24 [14]. Recently, it has been found that there is the same pathway in *Mycobacterium tuberculosis* and others [6]. We are interested in the trehalose synthase that catalyses this third pathway because it uses a very simple disaccharide (maltose) as substrate. All trehalose synthases reported so far have a very interesting characteristic: they all catalyze the reversible reaction of conversion between maltose and trehalose with equilibrium point at about 80% trehalose and 20% maltose, which is apparently in favor of trehalose formation. The question raised would be whether the trehalose synthase is ubiquitous among the microbiological kingdom, and if so, whether they share the same characteristic, especially whether they will have the same equilibrium point of above or they will all have great discrepancy.

Thermobifida fusca is an aerobic, thermophilic soil bacterium that has optimal growing temperature of 55 °C and is an important degrader of plant cell walls in heated organic material, i.e., compost heaps, manure piles and rotting hay [17]. We report here the gene cloning and expression in *E. coli* of an uncharacterized ORF, annotated as "glycosidase" in the GenBank database, from the genome sequences of *Thermobifida fusca* DSM43792. The enzyme was characterized on its optimal temperature and pH. Blasting search against GenBank database indicates that its amino acid sequence has less than 70% similarity to all previously reported trehalose synthases and it is a new trehalose synthase.

Materials and Methods

Materials

Thermobifida fusca DSM43792 was obtained from DSMZ (Braunschweig, Germany). *E. coli* JM109 with genotype {*e14⁻(McrA⁻) recA1 endA1 gyrA96 thi-1 hsdR17 (rk⁻ mk⁺) supE44 relA1(lac-proAB) [F′ traD36 proAB lacIqZ M15]*} was used for the routine cloning and *E. coli* BL21 [*F⁻ ompT hsdSB(rB⁻ mB⁻) gal dcm*] was used for the gene expression. Expression vector pSE380 was obtained from Invitrogen (Carlsbad, USA). Restriction enzyme, ligase, and LA *Taq* DNA polymerase were all obtained from TaKaRa (Shiga, Japan). Trehalose from Sigma (St. Louis, USA) was used as standard. All other chemicals were of analytical grade.

Bacterial growth and extraction of total DNA

Thermobifida fusca DSM43792 was inoculated into DSMZ medium No. 65 containing (g/L): glucose 4, yeast extract 4, malt extract 10, pH 7.2, and incubated in rotary shaker at 55 °C and 150 rpm for 24 h. 1.5 ml culture broth was centrifuged at 5000 rpm for 10 min to collect cells. Total DNA was extracted according to reference [18].

tres gene cloning and expression vector construction

Based on the sequence of the ORF (GenBank No. gi|23017523) annotated as "glycosidase" in GenBank, *tres* gene was cloned from the total DNA of DSM43792 using PCR amplification with sense primer 5′-GAG-CCATGGAGAAGTCGATGACCAC-3′, and antisense primer 5′-TGGAAGCTTTCAGGACCGCTGGTC-3′ (*Nco*I and *Hind*III restriction sites were underlined respectively) so that to be cloned into the expression vector pSE380 which contains the same restriction sites. PCR amplification conditions were as following: 94 °C 2 min, 94 °C 30 s, and 72 °C 2 min for 30 cycles, and 72 °C 10 min for extension. The resulting recombinant vector containing *tres* was named pSE380-*tres*-fu.

tres gene expression in *E. coli* BL21 and SDS-PAGE of target protein

pSE380-*tres*-fu was used to transform the competent cells of *E. coli* BL21, and a single colony was inoculated into 20 ml LB liquid medium supplied with 100 μg/ml ampicillin, then incubated in rotary shaker at 37 °C and 150 rpm. After the A_{600} of the cell density reached 0.6, IPTG was added at final concentration of 1 mM to induce the gene expression. After 18 h induction, 1.5 ml culture broth was centrifuged, the pellet was dissolved into 2×SDS-PAGE loading buffer and boiled for 10 min, centrifuged at 10,000 rpm for 2 min, and 5 μl super-

natant was used to run the SDS-PAGE.

Assay of trehalose synthase activity

1.5 ml broth was taken after IPTG induction and centrifuged at 5000 rpm for 10 min to collect cells. Cell pellet was resuspended in 1 ml disruption buffer containing 2 mg/ml lysozyme and 1% Triton X-100, incubated at 200 rpm, 37 °C for 12 h, and the suspension was centrifuged at 10,000 *g* for 10 min to clarify the enzyme solution. 300 μl supernatant was added to the same volume of 30% (*W*/*V*) maltose solution in 0.1 mM phosphate buffer, pH 6.5. 300 μl distilled water was added to the same substrate solution as control. The thoroughly-mixed mixture was incubated at 30 °C water bath with shaking for 20 h. The reducing sugar was assayed according to [19], using the control as 100% scale. One enzyme activity is defined as 1% decrease of reducing power of the mixture under above conditions. The trehalose content in the reacted mixture was quantified and further confirmed using HPLC (Agilent 1100).

Optimal temperature and pH

The enzyme activity was assayed as described above with temperature varied 8–45 °C, and pH varied in 5.0–9.0. The enzyme activity was measured either by monitoring the decrease of reducing power or the formation of trehalose with HPLC.

Results

tres gene cloning and expression vector construction

PCR product was analyzed by using 6% agarose electrophoresis and the band with correct size was clearly shown on the electrophoresis [Fig. 1(A)]. The PCR product was recovered from the gel and ligated into pSE380 after double restriction enzyme digestion of *Nco*I and *Hin*dIII. The recombinant vector pSE380-*tres*-fu was used to transform *E. coli* JM109 to propagate. The pSE380-*tres*-fu was extracted and again double-enzyme digested with *Nco*I and *Hin*dIII, the result confirms that the PCR product has been cloned into pSE380, as seen from the appeared band with correct size [Fig. 1(B)]. DNA sequencing was performed to verify the authenticity of the gene sequence before pSE380-*tres*-fu was used to transform *E. coli* BL21 to express the target protein.

SDS-PAGE of expressed product

E. coli BL21 was transformed with pSE380-*tres*-fu to express the *tres* gene. The soluble proteins in the cell were analyzed with SDS-PAGE [26] after an IPTG induction of 18 h. A clearly visible band is observed with the same molecular weight corresponding to those estimated from deduced amino acid sequence (Fig. 2), the product accounts for about 15% of total soluble cell proteins, measured with thin-chromatography scanner. It is very interesting to observe this fairly high percentage of total cell proteins, since enormous investigations have previously shown that under normal conditions, genes from archaebacteria, especially those with extreme high GC or AT content, will be poorly expressed in *E. coli* [24].

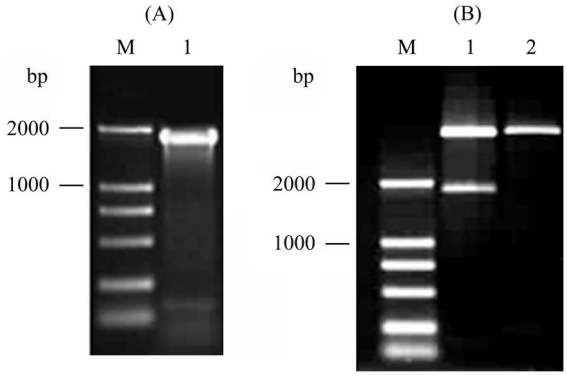

Fig. 1 Agarose electrophoresis of *tres* gene cloned from *T. fusca* genome by PCR

(A) Enzyme digestion picture of subcloning in *E. coli* JM109. M, DNA marker; 1, PCR product. (B) M, DNA marker; 1, the *tres* gene cut out from pSE380 by *Nco*I and *Hind*III double enzyme digestion; 2, pSE380-*tres*-fu.

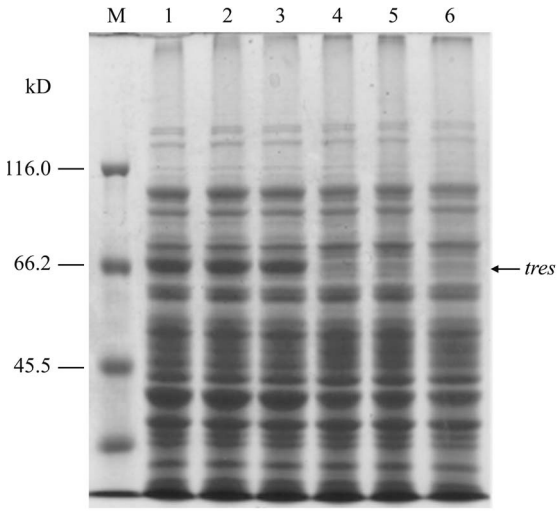

Fig. 2 SDS-PAGE of expressed product of *tres*

Arrow shows the position of *tres* protein which corresponds to the deduced molecular weight. M, protein standard marker. The experiments were repeated three times. 1, 2, and 3, from *E. coli* BL21 transformed with pSE380-*tres*-fu; 4, 5, and 6 from BL21 control.

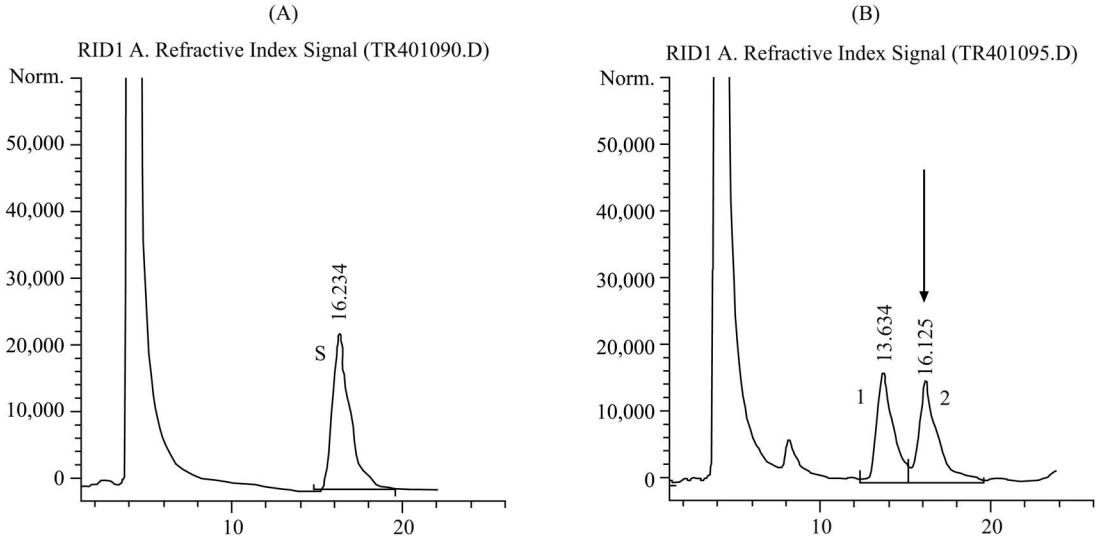

Fig. 3 HPLC analysis of 24 h enzyme reaction with 15% maltose as substrate

The reaction solution was diluted 20 times and 10 μl was loaded onto the column. Running conditions were 30 °C, 46 bars pressure, with acetonitrile:water=84:16 as mobile phase. (A) The standard trehalose shown as peak S. (B) Reacted sample and peaks. 1, maltose; 2, trehalose.

Enzyme characterization

After 20 h reaction, the supernatant was inspected for the presence of trehalose using HPLC (Fig. 3). From the chromatography it could be seen that about 50%–60% of the substrate is converted into trehalose under optimal conditions. HPLC is considered to be more reliable in the assay of enzyme activity since it can accurately measure the amount of product formation, but it is time consuming and the procedure is more tedious compared with reducing sugar assay. Two methods were compared by plotting their values in order to see whether they correlate well. The result indicates that in the range of 0%–55% of reducing power, the two methods correlated in good agreement, suggesting that the reducing power assay could be used routinely for estimation of trehalose synthase (Fig. 4). The reactions were set at different temperatures ranged in 8 °C–45 °C using a gradient PCR amplifier (Eppendorf). The optimal temperature is found to be about 25 °C (Fig. 5), similar to that of *Pimelobacter sp*. R48 [15], but at least 40 °C lower than that of *Thermus aquaticus* [16], which is surprising since *Thermobifida fusca* has an optimal growth temperature of 55 °C [17]. Enzyme was also tested against 0.1 mM phosphate buffer with different pH, and at 25 °C the optimal pH is around 6.5 (Fig. 6). The optimal pH is almost the same as those observed in the enzymes from *Pimelobacter sp*. R48 [15], and *Thermus aquaticus* [16].

Under the optimal conditions of 25 °C and pH 6.5, about

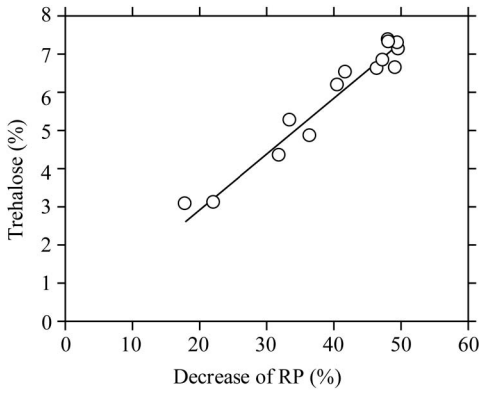

Fig. 4 Correlation of two enzyme assay methods

Substrate concentration was 15% maltose of 99% purity (*W*/*V*). Crude enzyme solution added was 100 μl out of total reaction volume 600 μl with 0.1 M phosphate buffer, pH 6.5, 25 °C. RP, reducing power.

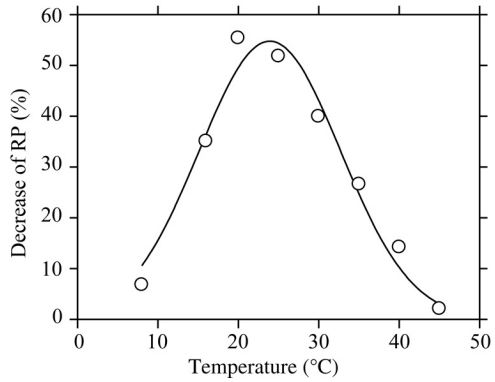

Fig. 5 Investigation on enzyme optimal temperature

The reaction conditions were the same as Fig. 4.

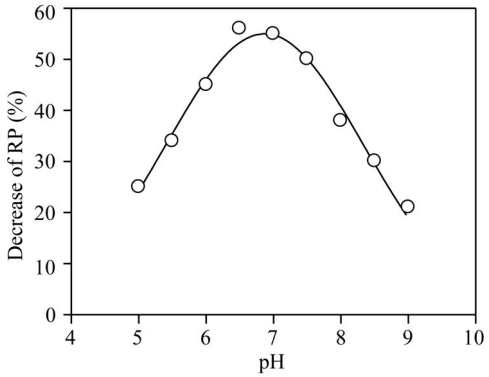

Fig. 6 Optimal pH for the enzyme was searched using phosphate buffer with pH in range of 5.0–9.0

The test conditions were the same as Fig. 4.

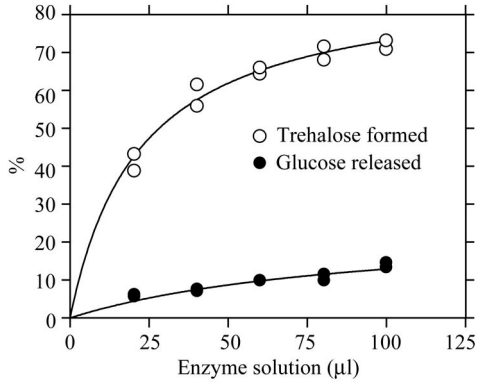

Fig.7 The trehalose and by-product glucose change with the increasing enzyme concentration

Trehalose synthesis is complied with typical model of saturated enzyme reaction, but glucose, the by-product of the enzyme reaction, is increasing linearly when the enzyme concentration is increased, (see Fig. 4 for the reaction conditions). The data were taken from duplicate experiments.

60% of substrate could be converted into trehalose by this enzyme, which was lower than those obtained by the same type of enzyme characterized before [15,16], which were in range of 75%–82%. We have observed some interesting results from the characterization of this enzyme, which were not reported on the other enzymes before. Firstly, it will divert a considerable amount of substrate, which could reach as much as 15%, into glucose, if enzyme concentration is increased (Fig. 7), or if the temperature was increased, e.g. from 25 °C to 37 °C (data not shown). Secondly, since it is observed that this enzyme will produce a considerable amount of glucose, we presumed that inclusion of high concentration of glucose in the reaction system may facilitate shifting the reaction direction towards the formation of trehalose, but in fact our experiment has indicated that it is not the case. To be opposite, addition of glucose into the reaction mixture would result in the decrease of trehalose formation (Fig. 8), i.e. increase of glucose concentration may inhibit the enzyme and thereby the final conversion of maltose.

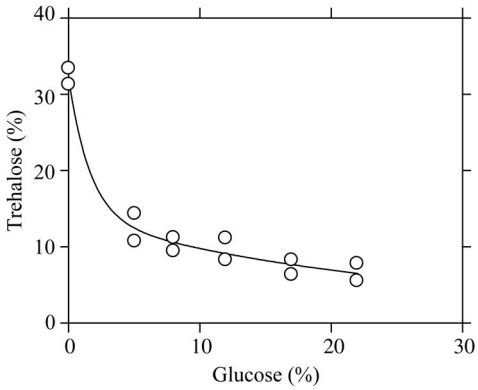

Fig. 8 Addition of glucose to the enzyme reaction caused retardation of trehalose synthesis

The substrate concentration was kept constant at 8% maltose, other reaction conditions were same as that in Fig. 4. The data were taken from duplicate experiments.

The amino acid sequence of *tres* was blasted against four other trehalose synthases whose functions have been experimentally validated and the sequences are publicly available (Table 1). The alignment was undertaken

Table 1 Pairwise similarities among the five trehalose synthases

Synthase	R48 (573 aa)		*T. aquat.* (963 aa)		*M. tuberc.* (601 aa)		*Brady.* (1098 aa)	
	Score	Identity	Score	Identity	Score	Identity	Score	Identity
T. fusca (610 aa)	0.943	69%	0.570	49%	1.000	68%	0.693	52%
R48 (573 aa)	–	–	0.539	51%	0.934	69%	0.663	56%
T. aquat. (963 aa)	–	–	–	–	0.568	49%	0.600	35%

The result of the running on the DiAlign Web Server (http://www.genomatix.de/). The score is of DiAlign, and the identity is with the amino acid sequence (in % of shorter sequence). The maximal score and similarity are underlined. *T. fusca*, *Thermobifida fusca*; R48, *Pimelobacter sp*. R48; *T. aquat.*, *Thermus aquaticus*; *M. tuberc.*, *Mycobacteria tuberculosis* [6]; *Brady.*, *Bradyrhizobium japonicum* USDA110 [20].

using DiAlign2 program implemented with a new algorithm featured by segment-to-segment local sequence searching and non-gap penalty alignment [21,22], which is different from the routine-used BLAST and PSI-BLAST algorithm [23]. The result of pairwise similarities is shown in Table 1 and the alignment for the five *tres* enzymes is shown in Fig. 9. For the *tres* of *T. fusca*, there are two segments, i.e. amino acid position 223–257 and 400–439, both with about 35 aa, which show highly conserved among these five microorganisms by having over 85% identity.

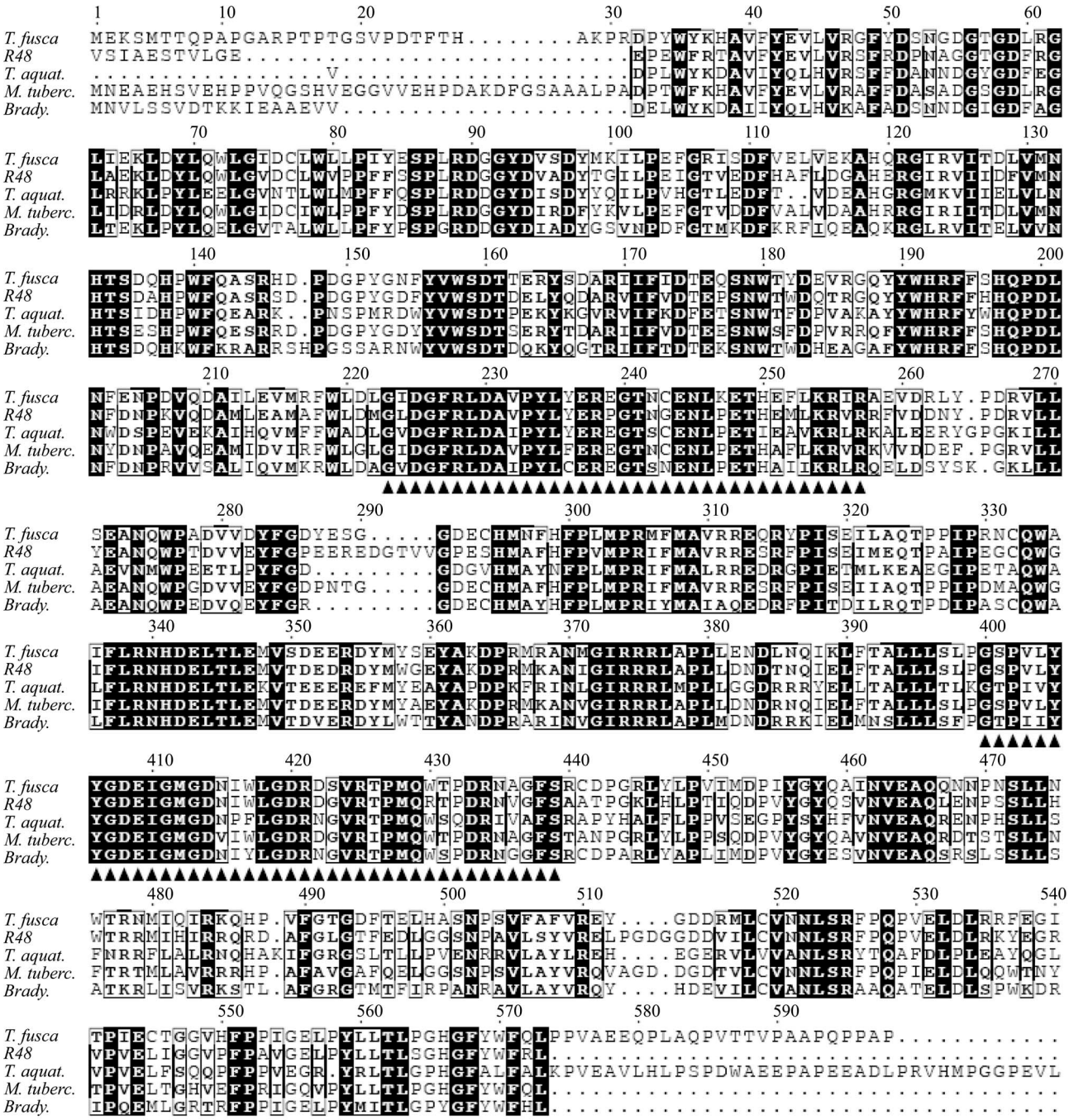

Fig. 9 Protein sequence alignment of trehalose synthases from five different microorganisms

Alignment was run on DiAlign server using algorithm as described in [21,22]. The graph was prepared with ESPript running on local SGI Octane. The best conserved regions in the alignment of the five proteins are marked with triangles, which correspond to amino acid positions of 223–257 and 400–439 of *T. fusca*. See Table 1 for the names of the microorganism strains.

Discussion

A new trehalose synthase has been found in the genome of *Thermobifida fusca* DSM43792. The ORF (open reading frame) was annotated as "glycosidase" in the GenBank database, and our experiment has identified this ORF as trehalose synthase with optimal temperature 25 °C and optimal pH around 6.5. Started with 15% maltose, the enzyme could convert about 55%–65% substrate into trehalose, which was lower than similar enzymes from *Pimelobacter sp*. R48 [15] and *Thermus aquaticus* [16] reported before. The enzyme was not inhibited by 5 mM of heavy metals including Cu^{2+}, Zn^{2+} and Mn^{2+} (data not shown). Isolation of trehalose synthase from the cultured cells of natural strain of *T. fusca* DSM43792 has proved to be unsuccessful since the cell homogenate has shown no enzyme activity even the supernatant is concentrated for 500 times (data not shown).

Sequence alignment of five trehalose synthases reported so far indicates that this enzyme shares 69% sequence similarity with the one from *Pimelobacter sp*. R48, and 68% with that from *Mycobacteria tuberculosis*. All these three synthases have similar optimal temperature and pH, but their enzyme turnovers are rather different with the one from *Pimelobacter sp*. R48 being the highest, which reaches a reaction equilibrium point of about 80% trehalose and 20% maltose, and a small amount of glucose (less than 3%, a by-product) released. In contrast, the enzyme reported here will reach the same equilibrium state with only about 60% trehalose and 25% maltose, and as high as 10%–15% glucose is released. From the recent report on the investigation of catalytic mechanism of the trehalose synthase [14], it can be clearly seen that glucose is the normal by-product of the enzyme, and at least for the one from *Thermus caldophilus* GK24, this diverted reaction pathway is irreversible. This explains why the addition of glucose into the reaction system does not shift the reaction towards the formation of either trehalose or maltose. Interestingly, inclusion of glucose in the reaction mixture will dramatically retard the enzyme catalytic formation of trehalose, and what type of inhibitor the glucose acts as remains to be further studied.

The information from the sequence alignment by the newly developed program DiAlign could be quite illustrative since its score is based on segment-to-segment alignment [21]. If a segment of an enzyme molecule is highly conserved among the organisms involved, it could indicate that this segment may be functionally important for this enzyme group since the evolution of protein molecules has shown to comply with a general rule: the functionally important areas in protein 3D structure such as substrate binding and catalytic domains will be well conserved over millions of years [25]. Two highly conserved segments 223–257 aa and 400–439 aa in the trehalose synthase molecule of *T. fusca* could be two functional motifs for this group of enzymes. Since there is still no three dimensional structure of trehalose synthase available in the database so far, building a 3D model for *tres*-fu is difficult at presence because of lacking the templates. Nevertheless, it is possible to use the site-mutagenesis to pinpoint the critical amino acids in the enzyme molecules, started with the two conserved segments above. But a full dissection of this enzyme structure and illustration of its catalytic mechanism can only rely on the 3D structure information obtainable through X-ray diffraction or nuclear magnetic resonance (NMR). Until then, many questions could be partially answered such as why this enzyme can only stand to about 30 °C while its original host is able to thrive well at 55 °C, why it diverts such large amount of substrate into the by-product glucose, and how to engineer the enzyme molecule to increase its thermostability.

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