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Directed Evolution of Operon of Trehalose-6-phosphate Synthase/Phosphatase from *Escherichia coli*

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Trehalose is a nonspecific protective agent for biomacromolecules. Trehalose-6-phosphate synthase (OtsA)/phosphatase (OtsB), which is encoded by the gene operon *otsBA* located at −42 of the *Escherichia coli* genome, is the main enzyme system that catalyzes the synthesis of trehalose in *E. coli*. We cloned the operon and modified it by directed evolution. Unlike in the previously reported work, we modified the whole operon and screened the positive mutant simultaneously. Thus we believe that the gene complex solves the negative effects between two enzymes if one of them diversifies its structure or functions and finds the form most suitable for trehalose synthesis. It is thus mimics the natural process, in which the functional improvement of organisms is related to alterations in coordinated enzymes. The evolution procedure was carried out in a sequence of error-prone PCR, shuffling PCR, and then strict screening of the mutants. After screening of a library of more than 4000 colonies, about 15 positive colonies were analyzed, resulting in a higher concentration of trehalose than control. One of them, *E. coli* TS7, shows 12.3-fold higher trehalose synthesis ability than *E. coli* DH5α. In contrast, we introduced the cDNA sequence of the *tps1* gene from *Saccharomyces cerevisiae*, which has 54% identity with the gene *otsA*, as one of the templates in shuffling PCR. By hybrid evolution and screening, we obtained 10 positive colonies with higher concentrations of trehalose than control. *E. coli* TS22 appears to have 5.3-fold higher trehalose synthesis ability than *E. coli* DH5α and 1.6-fold more than *E. coli* DEF3(pOTS11). This result demonstrated that coevolution and hybrid evolution, as powerful protocols in protein engineering, are effective in modifying enzyme. It indicates that repeating the process of genomic evolution in nature is feasible.

Directed evolution, a new strategy of protein engineering, has been widely used in recent years to design enzymes with improved activities or properties (1, 2). There are many successful examples in the list, such as Crameri's work on *para*-nitrobenzyl esterase (3), Beaudry's work on ribozyme (4), Shao's work on *subtilisin* E (5), and Zhang's work on aspartase (6, 7). In their work, the sequence of gene fragments was randomly mutated and recombined *in vitro* to construct a large mutant library. The library was then screened under conditions to procure the target characters. It is not necessary to know the detailed three-dimensional structure and the catalytic mechanism before we decide to re-form a protein with this method. The efficient protocol makes it possible for us to obtain various novel phenotypes in one step. It is just like the molecular evolution progress in nature. We found that single genes coded all these enzymes. However, as we know, a phenotype change is not always the contribution of one gene modification. The directed evolution method does not work in a more complex expression system such as yeast (not published). Also, it cannot duplicate natural evolutionary events, in which related proteins were altered in coordination.

Trehalose [*O*-α-D-glucosyl-(1 → 1)-α-D-glucoside] is a disaccharide combined with two glucoses with (α, α)-1 → 1 glucosidic bonds. It is a stress metabolite in various organisms (8). It has been suggested that trehalose can nonspecifically protect biological macromolecular from destruction. There are three main ways for organisms to synthesize trehalose *in vivo*. The first way is catalyzed by trehalose synthase (9), which converts maltose into trehalose by transglycosidation. The second way is catalyzed by trehalose phosphorylase to synthesize a trehalose molecule from a molecule of β-glucose 1-phosphate and a molecule of glucose (10). The last is the most common way adopted by microbes and depends on a two-enzyme system requiring a high-energy intermediate of glucose metabolism as substrate. Trehalose-6-phosphate synthase (TPS) transfers a glucose unit from UDP-glucose to glucose

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6-phosphate to yield trehalose 6-phosphate (Tre6P), and trehalose-6-phosphate phosphatase (TPP) hydrolyzes the Tre6P into trehalose and P_i. In *E. coli*, the two enzymes are coded by the operon *otsBA* (11, 12). In order to study the relationship at the sequence level of the two overlapping genes and to construct an engineering microbe to produce trehalose, we have cloned the operon and mutated two enzymes simultaneously by directed evolution, which we call coevolution. Another problem in directed evolution of the *E. coli otsBA* operon is that the potential for enzymatic activity enhancement is limited. The number of base pairs of the operon is definite, and after billions of years of natural selection, most of the evolutionary possibilities have been already eliminated. To increase the potential of evolution, we have adopted a hybrid evolution strategy. Because of homogeneity, the truncate product will bond to the heterogeneous template and add foreign sequence when extended. This evolution protocol was named hybrid evolution because the templates come from different sources.

MATERIALS AND METHODS

Reagent and primers. Restriction enzymes, *Taq* DNA polymerase, and *Pfu* polymerase were purchased from MBI. Reagents and primers (POTS1, 5′-GTGCGAGCTCATGTCTGTAAAGCGCGTTC-TGCG-3′; POTS2, 5′-GTGAGGTCGACGTGCTGTTAGTTCCACT-TAGG-3′; PTPS1P, 5′-GCGTGAGCTCCAAAGCAGGCTAACAAAC-TAGG-3′; PTPS1M, 5′-GCGGGATCCAGAAACCGGAACCAGGAA-TAGAAG-3′) were purchased from Shanghai Sangon, China. The first primer sequence of the oligonucleotide was derived from the sequence reported by Kaasen *et al.* (12). The underlined sequences are *Sac*I and *Sal*I restriction sites for POTS1 and POTS2, respectively. The second primer sequence was derived from the sequence reported by Bell *et al.* (13). The underlined sequences are *Sac*I and *Bam*HI restriction sites for PTPS1P and PTPS1M, respectively.

Deficient strain of trehalase. The host that lacks trehalose hydrolysis activity was prepared by UV mutation. *E. coli* strain DH5α was put under 253-nm UV light for 10 min and then spread on a LB medium plate and cultured at 37°C. When bacterial colonies grew to 1 mm in diameter, the plate was imprinted to TST solid medium (0.5% tryptone, 0.5% NaCl, 1% trehalose, and 2% agar) and TSG solid medium (0.5% tryptone, 0.5% NaCl, 1% glucose, and 2% agar). The imprinted plates were incubated at 37°C for 16 h. Those colonies that could grow on TSG plate but could not grow on TST plate were chosen.

Trehalose determination. Bacterial strains be used for trehalose determination were cultured for 16 h at 37°C in LB medium. Then the cells were collected by centrifugation at 4°C. For trehalose measurements, the pellets were washed three times with distilled water at 4°C. The pellets were suspended in three times their volume of 0.5 M trichloroacetic acid and extracted for 30 min at room temperature. After centrifugation, a second extraction of the pellets was performed for 30 min at room temperature with a similar volume of 0.5 M trichloroacetic acid (14). The combined supernatants from the two extractions were assayed by phenol/sulfuric acid methods (15). The sample was mixed with 5% phenol and sulfuric acid sequentially at the ratio 1:1:5. The mixtures were incubated at room temperature for 5 min and then analyzed at 470 nm (on a Shimadzu UVPC-2501 spectroscope). In each experiment, we verified that trehalose was indeed the only carbohydrate extracted by HPLC.

Cloning of the operon otsBA. The genomic DNA of *E. coli* DH5α was prepared as described by Ausubel *et al.* (16). The operon *otsBA* was isolated by PCR with *Pfu* DNA polymerase. The oligonucleotides POTS1 and POTS2 were used as primers. The template was purified genomic DNA of *E. coli* DH5α. The amplified fragment was digested with restriction endonucleases *Sal*I and *Sac*I at 37°C for 2 h. Then the fragment was ligated to plasmid pUC18, which was cleaved with the same enzymes. The recombinant plasmid was named pOTS11. Cyclic ligated products were transformed into *E. coli* DH5α. The recombinant plasmid was screened by blue/white selection (17). Cloned fragment was confirmed by restriction digestion analysis.

Recombinant plasmid pTPS15. The *S. cerevisiae tps*1 gene was amplified from *S. cerevisiae* mRNA using the reverse transcription polymerase chain reaction (RT-PCR). The pair of primers is PTPS1P and PTPS1M. The amplified gene inserted into pUC18 by restriction enzymes *Sac*I and *Bam*HI. The connected mixtures were transformed into *E. coli* DH5α The recombinant plasmid was screened by blue/white selection. Sequence assay proved that the fragment is the gene of *S. cerevisiae tps*1.

Strategy for coevolution. Random mutagenesis was carried out using error-prone PCR (5) with oligonucleotides POTS1 and POTS2. The recombinant plasmid was used as PCR template. The amplified PCR products, which were about 2.1 kb in length, were purified by gel electrophoresis and extracted using Silver Beads DNA Extraction Kit (Sangon, China). The purified DNA fragments were used as PCR template for another round of error-prone PCR. DNA shuffling is also accomplished by PCR (shuffling PCR). The amplified fragment mixture containing *otsBA* mutant operon was isolated and used as PCR template. The new amplifying process was carried out with the same two primers in a PCR of 10 cycles of 95°C 60 s, 50°C 60 s, 72°C 20 s, → 20 cycles of 95°C 60 s, 52°C 70 s, 72°C, 20s, → 70 cycles of 95°C 60 s, 53°C 90 s, 72°C 30 s, followed by 72°C for 10 min. The final PCR products were ligated into pUC18 vector. The ligation mixture was then transformed into competent cells of *E. coli* strain with the phenotype (*tre*$^-$) to generate a library of *otsBA* mutant operons.

Strategy for hybrid evolution. The purified recombinant plasmid DNA (including *otsBA* operon) was used as one of the shuffling PCR templates for hybrid evolution; the other template was recombinant plasmid DNA that included the sequence of *tps*1 cDNA and mixed *ots*BA DNA and *tps*1 DNA in the ratio of 1 to 5. The shuffling PCR method is similar to the coevolution PCR method. The final PCR products were ligated into pUC18 vector. The ligation mixture was then transformed into competent cells of *E. coli* strain with the phenotype (*tre*$^-$) to generate a library of *otsBA/tps*1 recombinant operons.

Screen of positive strains. The positive strains were imprinted on a TS medium plate (0.5% tryptone, 0.5% NaCl, and 2% agar). After incubation for 16 h and colony growth to 1 mm in diameter, the plate was immersed into 0.5 M trichloroacetic acid for 5 min at room temperature. Then the plate was treated with 0.4 ml 5% phenol solution and 2 ml sulfuric acid. The colonies gave different colors from light brown to deep black. Colonies on the original plate that showed black spots were inoculated into to 2 ml LB liquid medium and cultured at 37°C for further analysis.

RESULTS AND DISCUSSION

An organism is a more delicate and complex system than we have ever though. For some enzymes involved in the process of cell metabolism, we cannot always change the phenotype of the cell by modifying one protein. A series of factors related to the process must be altered synchronously to get a different metabolism pathway. We could easily enhance TPS and TPP expression levels by random mutagenesis or other ge-

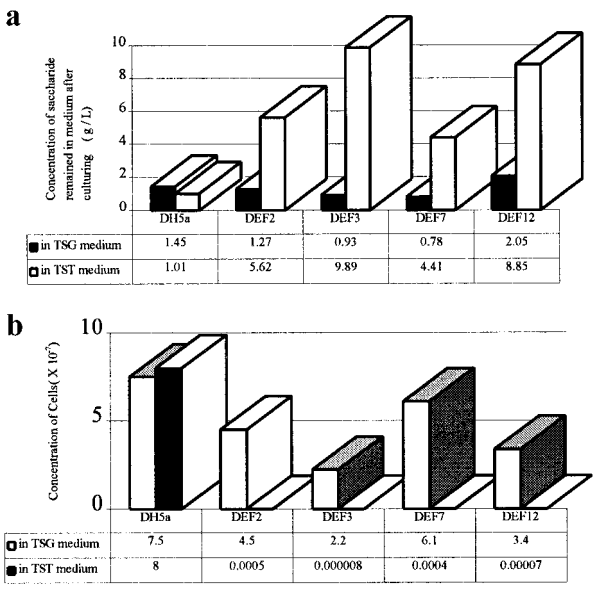

FIG. 1. (a) Growth ability and (b) saccharide metabolism of different strains of *E. coli* in TSG and TST media. All data were collected after cell culturing at 37°C for 16 h.

netic protocols. But it is difficult for us to control the balance between synthesis and hydrolysis of T6P *in vivo.*

If we modify the two genes at the same time and screen the variants in a library which contains all evolution potentials, we could find the exact one that has the most cooperation of the two enzymes—enzymome. It is not necessary to analyze the properties of the two enzymes. We should consider them separately, since it is not our original thought to look at the two enzymes as a whole.

Screening mutant strain of E. coli with phenotype tre$^-$. Under conditions of low pressure, bacterial cells will produce trehalase to hydrolyze the overproduced trehalose. We cannot measure the activity changes of trehalose synthesis accurately if we cannot inhibit the function of trehalase. Using the method described under Materials and Methods, we obtained an *E. coli* strain that is trehalose utility defective. About 20 colonies grew on a TSG plate but not on a TST plate. Among them, 4 strains grew much slower than others in TST liquid medium for 16 h at 37°C. The concentration of trehalose in the medium changed little after 16 h culturing (see Fig. 1). And one of them, DEF3, nearly cannot utilize trehalose at all. But it can grow on medium in which glucose was used as the carbon source, as well as that for *E. coli* DH5α. We named the strain *E. coli* DEF3 (*tre*$^-$) and used it as host for foreign gene in later work.

Coevolution of the operon otsBA. We developed a novel method of DNA shuffling. The main idea of the method is to reduce the extension time and let the truncated products switch the template in the following cycle (Fig. 2). Thus, the final products of PCR reaction consisted of all possible combinations of the point mutations introduced by error-prone PCR. Unlike the strategy of StEP designed by Zhao *et al.* (18), the amplifying process was performed by a pair of primers instead of single one. Two primers allowed shuffling and amplifying in one simple PCR step to reduce the amount of template. Nearly all amplified fragments were recombined products. This makes the screening process more efficient because we need not deal with a large number of parent variants.

Figure 3 shows the extension process of shuffling PCR. At the 40th cycle, there are large quantities of nonspecific amplification products ranging from about a hundred to several thousands. The shorter products were much less at cycle 80, when the truncated PCR products were elongated.

More than 4000 colonies were screened. Among them, 15 colonies showed a deeper black than others. The trehalose content of the cells was determined (see Table 1). Strain TS7 exhibited 12.3-fold higher carbohydrate content than *E. coli* DH5α and 3.70-fold higher than *E. coli* DEF3 (pOTS11). Figure 4 shows the protein constitution of *E. coli* strains DEF3 (pOTS11) and TS7. The expression levels of foreign proteins are similar in these two strains. We can derive from the fact above that the rise of trehalose content is mainly contributed by the increasing activity of the enzymome. Further TLC test (result not shown) confirmed that trehalose was the only saccharide stored by the cell.

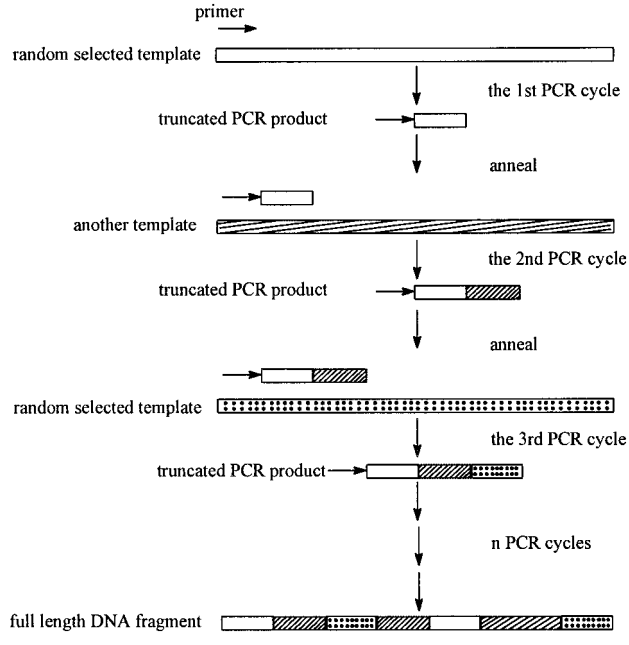

FIG. 2. Schematic illustration of DNA shuffling by PCR.

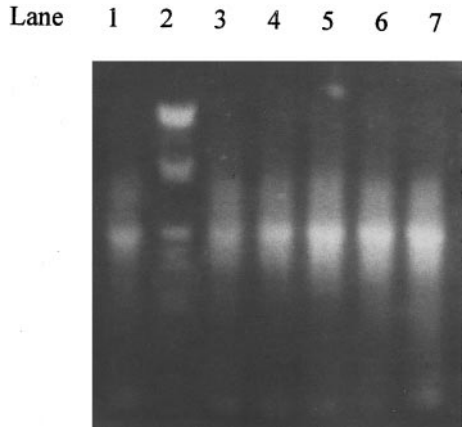

FIG. 3. Process of shuffling PCR. Lane 1, final shuffling PCR product; lane 2, DNA molecular weight marker (λ/*Eco*RI, *Hin*dIII); lanes 3–7, truncated PCR products collected at cycles 80, 70, 60, 50, and 40; the amount of sample added to the gel is increased step by step with the cycles decreasing (1.0% agarose gel).

Hybrid evolution of otsBA operon and tps1 gene. The method of hybrid evolution is similar to coevolution. But the two templates in the hybrid evolution differ more from each other than in the coevolution. So the nonspecific amplified product has increased obviously in quantity and distribution (result not shown). For inserted allogenetic gene, we selected the two templates, *otsBA* operon and *tps*1 gene, in the ratio of 1 to 5.

We screened more than 1000 colonies. Ten colonies were a deeper black than others. The trehalose content of strains was determined (see Table 1). Strain TS22 showed 5.3-fold higher carbohydrate content than *E. coli* DH5α and 1.60-fold higher than *E. coli* DEF3 (pOTS11). But further SDS–PAGE analysis of the protein constitution of *E. coli* strains DH5α, DEF3 (pOTS11), and TS22 (result not shown) proved that the quantity of the allogenetic gene expression was not higher than the wild type. We thought the increase of trehalose because of the improvement of enzyme activity.

TABLE 1

Trehalose Content of Cells[1]

E. coli strain	Trehalose content (mg/g cells)
DH5α	1.04
DEF3	2.19
DEF3(pOTS11)	3.45
TS1	4.75
TS2	5.60
TS3	4.24
TS4	7.61
TS5	8.56
TS6	8.32
TS7	12.77
TS8	6.34
TS9	9.13
TS10	12.10
TS11	7.23
TS12	5.43
TS13	9.06
TS14	10.13
TS15	10.15

[1] All data were collected after 16 h culturing.

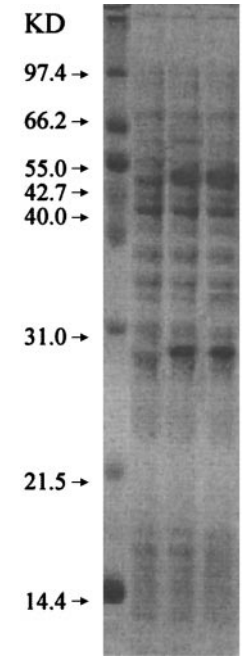

FIG. 4. Protein constitution of *E. coli* strains DH5α (lane 2), DEF3 (pOTS11) (lane 3), and TS7 (lane 4). Lane 1 is marker (mid-range protein MW marker). The two overexpressed proteins, which are about 29 and 54 kDa, correspond to the molecular weights of *OtsB* and *OtsA*, respectively (12).

From our work, we confirmed that hybrid evolution is an effective tool in protein engineering and molecular evolution engineering. Hybrid evolution is an important flight in the course of natural evolution. This phenomenon is rare in nature but every occurrence was accomplished with an important change in the biological universe, such as lichen (19) and *Rhizobium leguminosarum* (20).

CONCLUSION

Screening efficiency is the hinge of directed evolution. But until now, there has been no fast and sensitive approach to distinguish the differences in trehalose content among a large number of colonies. It was not our intention to inspect all the variants in the mutant library. Despite this, we have obtained a mutant with 12.3-fold increase in carbohydrate by coevolution. Another reason that we cannot obtain a variant with a higher enhancement of trehalose production is

probably because a high concentration of trehalose is a signal of stress and the metabolism level of cell is reduced.

The result not only is beneficial for industrial production of trehalose, but also demonstrates that modifying a group of related enzymes at the same time by coevolution is feasible. It also manifests that an organism that is inert in evolution has evolutionary potential. The stabilization of genotype is based on the stabilization of environment, and the evolutionary direction is determined by the type of environmental change. The theory of evolution is verified by some aspects of this study.

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