Random Transfer of *Ogataea polymorpha* Genes into *Saccharomyces cerevisiae* Reveals a Complex Background of Heat Tolerance

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Abstract: Horizontal gene transfer, a process through which an organism acquires genes from other organisms, is a rare evolutionary event in yeasts. Artificial random gene transfer can emerge as a valuable tool in yeast bioengineering to investigate the background of complex phenotypes, such as heat tolerance. In this study, a cDNA library was constructed from the mRNA of a methylotrophic yeast, *Ogataea polymorpha*, and then introduced into *Saccharomyces cerevisiae*. *Ogataea polymorpha* was selected because it is one of the most heat-tolerant species among yeasts. Screening of *S. cerevisiae* populations expressing *O. polymorpha* genes at high temperatures identified 59 *O. polymorpha* genes that contribute to heat tolerance. Gene enrichment analysis indicated that certain *S. cerevisiae* functions, including protein synthesis, were highly temperature-sensitive. Additionally, the results confirmed that heat tolerance in yeast is a complex phenotype dependent on multiple quantitative loci. Random gene transfer would be a useful tool for future bioengineering studies on yeasts.

Keywords: *Saccharomyces cerevisiae*; cDNA library; heat tolerance; random gene transfer; *Ogataea polymorpha*

1. Introduction

Horizontal gene transfer is a rare evolutionary event in yeast, in which functional genes are acquired from other species [1]. Gene transfer among eukaryotes is a relatively rare event that is limited by unknown barriers [2–4]. Genome analysis revealed that the budding yeast *Saccharomyces cerevisiae* has acquired several genes from bacteria, such as those encoding metabolite enzymes and transporters [5–7].

Artificial random gene transfer is a technique used in conventional genetic engineering to identify valuable genes, such as those useful for metabolic engineering [8]. In addition, the technique can help investigate the genetic background of quantitative traits, as many loci, each with small effects, contribute to heat tolerance [9,10]. Transfer of a gene pool would provide a list of genes that contribute to heat tolerance in the host yeast, and this list of genes will help elucidate the genetic complexity behind the heat tolerance phenotype.

In this study, we used a cDNA library for a random gene transfer experiment [11]. The cDNA library was constructed from the mRNA of a methylotrophic yeast, *Ogataea polymorpha*, and then introduced into *S. cerevisiae*. *Saccharomyces cerevisiae* is an industrial host for bioethanol production. Since improved heat tolerance reduces the costs required for cooling during fermentation [12], many genetic analyses and adaptive evolutionary studies have been performed [13–15]. *Ogataea polymorpha* was selected because it can grow at temperatures close to 50 °C and is one of the most heat-tolerant species among yeasts [16,17]. Under high temperatures, screening the *S. cerevisiae* population identified 60 colonies showing improved heat tolerance and the corresponding *O. polymorpha* genes.
responsible for heat tolerance. Additionally, the list of genes uncovered the *S. cerevisiae* functions that are sensitive to high temperatures.

2. Materials and Methods

2.1. Strains, Plasmids, and Yeast Transformation

The yeast strains and plasmids used in this study are listed in Table 1. Plasmids were derived from pGK413, pGK414, or pGK416, in which gene expression is controlled by the PGK1 promoter [18]. For the construction of cDNA libraries, *O. polymorpha* BY4329 cells were cultured till the exponential growth phase in 5 mL of yeast extract-peptone-adenine-dextrose (YPAD) medium and then harvested by centrifugation at $12,000 \times g$ for 5 min. mRNA was extracted from the cells using the Ribo-Pure Yeast Kit (Thermo Fisher Scientific, Waltham, MA, USA). Two overlapping regions for the In-Fusion method were added to the three plasmids using the inverse PCR method with pGK413, pGK414, and pGK416 as the templates and the primers pGK_inv_f (TCTCATCGTACCCCGAAATAAATT) and pGK_inv_r (AAGCATGCTGACGAAGTTTTATATTTGTTG) [11]. A cDNA library was constructed from the mRNA mixture and the amplicon of inverse PCR, using the In-Fusion SMARTer Directional cDNA Library Construction Kit (Takara Bio, Inc., Shiga, Japan). The In-Fusion mixture was introduced into *Escherichia coli* HST08 competent cells (Takara Bio, Inc.) by electroporation at 25 $\mu$F and 2 kV. The pulse controller was set to 200 $\Omega$ using a GenePulser (Bio-Rad Laboratories, Hercules, CA, USA). Ampicillin-resistant cells were recovered from agar plates, from which the plasmid pools were prepared. The growth conditions, DNA-related techniques, and the lithium-acetate method for transformation have been described previously [19].

Table 1. Strains and plasmids used in this study.

| Strain Name | Genotype | Source |
|-------------|----------|--------|
| *Ogataea polymorpha* BY4329 | Leu1-1 | Obtained from NBRP Yeast |
| *Saccharomyces cerevisiae* YPH499 | *MATa*, *ura3-52 lys2-801_amber ade2-101_ochre trp1-D63 his3-D200 leu2-Δ1 | Thermo Scientific |
| TT01 | YPH499 (pGK416_BY4329 cDNA library) | This study |
| TT02 | YPH499 (pGK413_BY4329 cDNA library) | This study |
| TT03 | YPH499 (pGK414_BY4329 cDNA library) | This study |
| TT01c | YPH499 (pGK416) | This study |
| TT02c | YPH499 (pGK413) | This study |
| TT03c | YPH499 (pGK414) | This study |
| *Escherichia coli* DH5α | *deoR* endA1 gyrA96 hsdR17 (rk-mk+) recA1 relA1 supE44 thi-1 Δ(lacZYA-argFV169) φ80lacZΔM15 F- F- endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, | |
| *Escherichia coli* HST08 | Δ(mrr-hsdRMS-mcrBC), ΔmcrA::λ– | |

Plasmids

| Plasmid | Description | Source |
|---------|-------------|--------|
| pGK413 | Yeast expression vector containing PGK1 promoter, origin, ARS4/CEN6 HIS3 marker, no expression (control plasmid) | [18] |
| pGK414 | Yeast expression vector containing PGK1 promoter, origin, ARS4/CEN6 TRP1 marker, no expression (control plasmid) | [18] |
| pGK416 | Yeast expression vector containing PGK1 promoter, origin, ARS4/CEN6 URA3 marker, no expression (control plasmid) | [18] |

2.2. Culture Conditions

All strains were cultured in YPAD medium (1% Bacto yeast extract, 2% Bacto peptone, 2% glucose, and 0.004% adenine) and synthetic dextrose (SD) medium (0.67% yeast nitrogen base without amino acids and 2% or 0.5% glucose, as necessary, 0.006% leucine, 0.003% lysine hydrochloride, 0.002% histidine, 0.004% adenine, 0.004% tryptophan, and 0.002%
uracil). Yeast cells grown on the agar plate were cultured in 5 mL of SD medium containing the required amino acids overnight at 30 °C and 150 rpm. To screen for cell growth, the transformants were cultured overnight in SD agar medium containing amino acids at 39 °C or 39.5 °C in an incubator (TVA360DB, ADVANTEC, Tokyo, Japan).

2.3. Construction of Screening System for the Heat-Resistant Evolved Strain

Saccharomyces cerevisiae YPH499 was transformed with the cDNA library of O. polymorpha using the lithium-acetate method and then cultured for several days in SD agar medium to obtain colonies of transformants. From the original SD agar plates, replica plates were prepared on other SD agar plates using the replica plating method. The replicas were cultured at 39 °C or 39.5 °C. The plasmids in the selected transformants were extracted using the Easy Yeast Plasmid Isolation Kit (Takara). Each plasmid was introduced into E. coli HST08 competent cells and cultured in L medium containing 5 mL ampicillin. Plasmid purification was performed using LaboPass Mini (Hokkaido System Science), and sequence analysis was performed using the PGK 5′ primer (TAGTTTTTCAAGTTCTTAGA) and PGK 3′ primer (CTATTATTTTAGCGTAAAGG). For each plasmid, the corresponding O. polymorpha gene was identified using the BLAST search function in the UniProt database, including O. polymorpha genome information (http://www.uniprot.org/ accessed on 13 April 2021) [17]. Saccharomyces cerevisiae orthologs were identified using the BLAST search of the Saccharomyces Genome Database (SGD, https://www.yeastgenome.org/ accessed on 13 April 2021). Gene enrichment analysis was performed using the over-representation analysis function of the WebGestalt web tool (http://www.webgestalt.org/ accessed on 13 April 2021) [20]. The Gene Ontology (GO) dataset of all O. polymorpha proteins was retrieved from the UniProt database. The Benjamini-Hochberg (GH) method was used to evaluate the false discovery rate (FDR).

2.4. Confirmation of Reproducibility by Spot Method

Transformants were inoculated on SD agar medium containing 20 g/L glucose and cultured at 30 °C for two days. A single colony grown on the plate was inoculated into a test tube containing 5 mL of SD medium and precultured at 30 °C and 150 rpm. The preculture solution was then centrifuged at 3000 rpm and 4 °C. The collected transformants were suspended in sterile distilled water. Suspensions (6 µL) were then spotted onto SD agar medium supplemented with the appropriate amino acids and incubated at 39 °C or higher for five days.

3. Results

3.1. Comparison of Vectors for Artificial Random Gene Transfer

For the construction of an O. polymorpha cDNA library, three CEN/ARS plasmid vectors (single copy-type), namely pGK416 (possessing URA3), pGK413 (possessing HIS3), and pGK414 (possessing TRP1), were employed [18]. Because the relationship between amino acid auxotrophy and heat tolerance was expected, three control strains possessing pGK416, pGK413, and pGK414 (strains TT01c, TT02c, and TT03c, respectively) were constructed from the S. cerevisiae YPH499 strain and cultured on agar plates to compare their heat tolerance phenotypes (Figure 1A,B). The TT01c and TT02c strains were able to grow at 39 °C but failed to grow at 39.5 °C. However, many colonies that grew at 39 °C showed an abnormally wet phenotype. The upper growth limit of TT03c was 38 °C (Figure 1C). These results showed that the amino acid auxotrophy of S. cerevisiae affected the heat tolerance of yeast for as yet unknown reasons.
3.2. Screening of Heat-Tolerant S. cerevisiae Strains Expressing O. polymorpha cDNA

A cDNA library was constructed from the O. polymorpha BY4329 strain. The cDNA fragments were inserted into pGK416, pGK413, and pGK414, which were then introduced into the S. cerevisiae YPH499 strain to produce three populations possessing O. polymorpha cDNA (TT01, TT02, and TT03, respectively). Approximately 400 colonies grew on each selection plate, and a replica plate was prepared using the replica plating technique. A total of 50–80 replica plates consisting of approximately 20,000–32,000 colonies were prepared for each population (TT01, TT02, and TT03).

The replica plates were incubated at 39.5 °C for the TT01 and TT02 populations and at 39 °C for the TT03 population. After one week, no colonies were obtained from the TT02 population. In contrast, 11 and 49 colonies were obtained on the replica plates of the TT01 and TT03 populations, respectively. No colonies were identified after additional screening at higher temperatures.

Following the collection of plasmid vectors from the 60 colonies, sequences of open reading frames of the cDNAs were determined to identify the corresponding O. polymorpha genes, S. cerevisiae ortholog genes, and their putative functions using the BLAST search of UniProt and SGD databases (Tables 2 and S1) [17]. Among the 60 colonies, an identical gene (OGAPODRAFT_52470, an ortholog of S. cerevisiae QCR8 ubiquinol-cytochrome c reductase subunit 8) was identified from two independent colonies (TT01-2 and TT01-8). The cDNAs obtained from four colonies (TT03-46, -47, -48, and -49) had poor homology to all S. cerevisiae ORFs (E-value < 1.0 × 10^{-5}), suggesting that these cDNAs were derived from O. polymorpha-specific genes.

Table 2. Annotation of O. polymorpha genes obtained from colonies of heat-tolerant S. cerevisiae expressing O. polymorpha cDNA (1).

| Colony ID | Gene ID of O. polymorpha (2) | S. cerevisiae Ortholog (3) | Functional Annotation of S. cerevisiae Ortholog |
|-----------|-----------------------------|---------------------------|-----------------------------------------------|
| TT01-1    | OGAPODRAFT_7331             | CAF20                     | cap-associated protein CAF20                   |
| TT01-2    | OGAPODRAFT_52470            | QCR8                      | ubiquinol-cytochrome c reductase subunit 8     |
| TT01-3    | OGAPODRAFT_16764            | ALD4                      | aldehyde dehydrogenase                         |
| TT01-4    | HPODL_02546                 | RPL16A                    | 60S ribosomal protein L16-B                    |
| TT01-5    | HPODL_00806                 | GUP1                      | acyltransferase                                |
| TT01-6    | OGAPODRAFT_17522            | THO1                      | SAP domain-containing ribonucleoprotein        |
| TT01-7    | OGAPODRAFT_12972            | HSP10                     | chaperonin GroES                               |
| TT01-8    | OGAPODRAFT_52470            | QCR8                      | ubiquinol-cytochrome c reductase subunit 8     |
| TT01-9    | HPODL_02610                 | CYT1                      | cytochrome c1, heme protein, mitochondrial     |
| TT01-10   | HPODL_04437                 | FRK1                      | serine/threonine protein kinase                 |
| TT01-11   | OGAPODRAFT_15309            | PAF1                      | RNA polymerase II-associated factor 1           |
| TT03-1    | HPODL_02637                 | GRS1                      | glycine-tRNA ligase 1, mitochondrial           |
| TT03-2    | HPODL_00026                 | NAP1                      | histone chaperone NAP1                         |
| TT03-3    | HPODL_05027                 | NAB2                      | mRNA-binding protein NAB2                      |
Table 2. Cont.

| Colony ID | Gene ID of *O. polymorpha* *(2)* | *S. cerevisiae* Ortholog *(3)* | Functional Annotation of *S. cerevisiae* Ortholog |
|-----------|----------------------------------|--------------------------------|--------------------------------------------------|
| TT03-4    | HPODL_03235                      | ERV25                          | p24 family protein delta-1                        |
| TT03-5    | HPODL_05028                      | RPS2                           | ribosomal 40S subunit protein S2                  |
| TT03-6    | OGAPODRAFT_25583                 | RIB3                           | 3,4-dihydroxy-2-butane-4-phosphate synthase RIB3 |
| TT03-7    | HPODL_03162                      | ACB1                           | long-chain fatty acid transporter ACB1           |
| TT03-8    | HPODL_01585                      | RAD4                           | DNA repair protein RAD4                          |
| TT03-9    | HPODL_00194                      | MRP7                           | mitochondrial 54S ribosomal protein VmL2         |
| TT03-10   | HPODL_02367                      | RPS31                          | ubiquitin-ribosomal 40S subunit protein S31      |
| TT03-11   | OGAPODRAFT_76806                 | CYT2                           | cytochrome c1 heme lyase CYT2                    |
| TT03-12   | OGAPODRAFT_92206                 | PSA1                           | mannose-1-phosphate guanylyltransferase          |
| TT03-13   | HPODL_01049                      | GRX6                           | glutathione-disulfide reductase GRX6             |
| TT03-14   | HPODL_00042                      | RPL7A                          | ribosomal 60S subunit protein L7A                |
| TT03-15   | HPODL_04105                      | RPL42A                         | ribosomal 60S subunit protein L42A               |
| TT03-16   | OGAPODRAFT_17069                 | PTI1                           | cleavage polyadenylation factor subunit PT1      |
| TT03-17   | HPODL_01073                      | ANB1                           | translation elongation factor elf-5A             |
| TT03-18   | HPODL_02594                      | MMF1                           | isoleucine biosynthesis protein MMF1            |
| TT03-19   | OGAPODRAFT_102344                | PGK1                           | 3-phosphoglycerate kinase                        |
| TT03-20 *(4)* | HPODL_02458                     | SOD2                           | superoxide dismutase SOD2                       |
| TT03-21 *(4)* | HPODL_02693                     | PFK26                          | 6-phosphofructo-2-kinase                        |
| TT03-22   | HPODL_02169                      | TAF9                           | transcription initiation factor TFIIID subunit 9 |
| TT03-23 *(4)* | HPODL_01966                     | RAD6                           | E2 ubiquitin-conjugating protein RAD6            |
| TT03-24   | HPODL_02705                      | RPL1A                          | ribosomal 60S subunit protein L1A                |
| TT03-25 *(4)* | HPODL_01497                     | ASC1                           | guanine nucleotide-binding protein subunit beta |
| TT03-26   | HPODL_01957                      | MET5                           | sulphite reductase (NADPH) subunit beta          |
| TT03-27   | OGAPODRAFT_75779                 | CEP3                           | centromere DNA-binding protein complex CBF3      |
| TT03-28   | HPODL_03364                      | RPL23B                         | ribosomal 60S subunit protein L23B               |
| TT03-29 *(4)* | HPODL_00942                     | RPP2B                          | ribosomal protein P2B                           |
| TT03-30   | HPODL_01497                      | ASC1                           | guanine nucleotide-binding protein subunit beta |
| TT03-31   | HPODL_02465                      | SER2                           | phosphoserine phosphatase                        |
| TT03-32   | OGAPODRAFT_74529                 | STE5                           | pheromone-responsive MAPK scaffold protein       |
| TT03-33 *(4)* | HPODL_03495                     | ACC1                           | acetyl-CoA carboxylase                           |
| TT03-34   | OGAPODRAFT_16247                 | DEG1                           | pseudouridine synthase                           |
| TT03-35   | OGAPODRAFT_76195                 | STM1                           | Uncharacterized protein                          |
| TT03-36   | OGAPODRAFT_17428                 | SLM1                           | phosphatidylinositol                             |
| TT03-37   | OGAPODRAFT_15585                 | RPS26A                         | 4,5-bisphosphate-binding protein                 |
| TT03-38   | HPODL_03366                      | SNF3                           | ribosomal 40S subunit protein S26A               |
| TT03-39   | HPODL_03527                      | IDPI                           | high-affinity glucose transporter SNF3           |
| TT03-40 *(4)* | OGAPODRAFT_7594                 | SOM1                           | isocitrate dehydrogenase (NADP(+))              |
| TT03-41   | HPODL_02149                      | ETR1                           | mitochondrial export protein Som1                |
| TT03-42   | HPODL_04585                      | MYO5                           | trans-2-enoyl-CoA reductase                      |
| TT03-43   | HPODL_01873                      | SBA1                           | myosin-5                                        |
| TT03-44 *(4)* | HPODL_01380                     | PRY2                           | hsp90 cochaperone SBA1                          |
| TT03-45   | HPODL_01021                      | RPS27B                         | sterol-binding protein                           |
| TT03-46   | HPODL_02251                      | n.d.                           | ribosomal 40S subunit protein S27B               |
| TT03-47   | HPODL_04413                      | n.d.                           | n.d.                                            |
| TT03-48 *(4)* | OGAPODRAFT_16908                | n.d.                           | n.d.                                            |
| TT03-49 *(4)* | OGAPODRAFT_15905                | n.d.                           | n.d.                                            |

*(1)* Full data are shown in Table S1. *(2)* *Ogataea polymorpha* genes were identified using the BLASTN function of UniProt. Partial nucleotide sequences were used as queries. *(3)* *Saccharomyces cerevisiae* orthologs were identified using the BLASTP function of SGD (E-value < 1.0 × 10⁻⁴). The full amino acid sequences of the *O. polymorpha* gene products were used as queries. *(4)* Corresponding strain was reconstructed for confirmation, as shown in Figure 2.

Functional categorization of the annotation list revealed that the transferred cDNAs encoded genes involved in various functions such as metabolism (for example,
Gene enrichment analysis was performed using the GH method to control the FDR. The results showed that genes encoding ribosomal proteins and other proteins involved in translation were overrepresented in the list of 59 *O. polymorpha* genes, with 11 (19%) and 9 (15%) cDNAs encoding genes related to the GO terms “structural constituent of ribosome” and “translation”, respectively (Table 3).

**Table 3.** Gene enrichment analysis of the list of 59 *O. polymorpha* genes.

| GO Term                      | False Discovery Rate (FDR) | Number of Matches |
|------------------------------|----------------------------|-------------------|
| structural constituent of ribosome | 0.00014579                | 11                |
| translation                  | 0.00028796                 | 9                 |
| ribosome                     | 0.047143                   | 6                 |

### 3.3. Reconstruction of Heat-Tolerant *S. cerevisiae* Strains

The *S. cerevisiae* strains listed in Table 2 were reconstructed to check the false-positive rate derived from the screening approach. For this purpose, 10 out of the 49 plasmid vectors were randomly selected from the TT03 populations and then introduced into the YPH499 strains. The heat tolerance of the reconstructed strains was investigated using a spot assay under high temperature conditions (Figure 2). Although 5-fold serial dilutions were employed to confirm differences in heat tolerance, we found that almost all reconstructed strains tended to be more tolerant to high temperatures (39°C) than the control strain (TT03c). These results suggest that the false-positive rate was low enough in the screening approach.

![Figure 2. Heat tolerance of reconstructed strains. The dilution series was employed to confirm differences in heat tolerance. Samples were withdrawn from cultures, and their OD_{600} values were adjusted to 20. Five-fold serial dilutions of these cultures were prepared in sterile distilled water, and 6 µL each of the cultures at OD_{600} = 20 and their dilutions were spotted onto SD plates without tryptophan. The plates were then incubated at 39 °C for 5 days and photographed.](image)

### 4. Discussion

In this study, we introduced a cDNA library derived from *O. polymorpha* into *S. cerevisiae*. Screening of the *S. cerevisiae* populations expressing the *O. polymorpha* cDNA library under high temperature conditions resulted in 60 colonies showing improved heat tol-
erance and identification of the *O. polymorpha* genes responsible for heat tolerance (Figure 2 and Table 2). These results reveal three aspects of the high temperature tolerance of *S. cerevisiae*.

First, we identified 59 candidate genes in *O. polymorpha* that contribute to heat tolerance. However, these results do not imply that the heat stability of proteins is derived from these genes. Since a strong promoter (PGK1 promoter) was used to express cDNA, a large amount of overexpressed proteins might have also contributed to heat tolerance. Further genetic and biochemical characterization is needed to examine the heat stability of the proteins expressed from the candidate genes. Moreover, this experiment failed to transfer all *O. polymorpha* genes to *S. cerevisiae* because the cDNA library used in this study was collected from *O. polymorpha* in exponential growth phase at 30 °C. More candidate genes are likely to be obtained using more comprehensive cDNA libraries prepared from *O. polymorpha*, for instance, under high temperature conditions.

Second, the genes identified in this study revealed the *S. cerevisiae* functions that are sensitive to high temperatures, because these functions were complemented by the expression of corresponding genes derived from *O. polymorpha*. Gene enrichment analysis showed that many of the *S. cerevisiae* proteins sensitive to high temperatures were ribosomal proteins and those involved in other steps of translation. Previous studies have reported that genes related to chaperonins [21], superoxide dismutase [22], ubiquitination [23], nitric oxide [24], H⁺-ATPase [25], and trehalose biosynthesis [26,27] were responsible for the heat tolerance of *S. cerevisiae*. While genes encoding chaperonin (TT01-7) and superoxide dismutase (TT03-20) were found, genes responsible for other functions such as H⁺-ATPase activity and trehalose biosynthesis were not found in the present study (Table 2). These results indicate that chaperonins, superoxide dismutase, ribosome, and translation may be additional targets for improving the heat tolerance of *S. cerevisiae*.

Thirdly, our results highlight that heat tolerance in yeasts is a complex phenotype that is controlled by multiple genes. This supports the idea that the improvement of heat tolerance in *S. cerevisiae* requires the expression of multiple heat-stable proteins. This study demonstrated that random gene transfer is a helpful laboratory evolution tool for investigating the genetic background of complex phenotypes, as well as for enabling future bioengineering studies.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jof7040302/s1, Table S1: Annotation of *O. polymorpha* genes obtained from colonies of heat tolerant *S. cerevisiae* expressing *O. polymorpha* cDNA.

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