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Quantitative target display: a method to screen yeast mutants conferring quantitative phenotypes by 'mutant DNA fingerprints'

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ABSTRACT

Whole genome sequencing of several microbes has revealed thousands of genes of unknown function. A large proportion of these genes seem to confer subtle quantitative phenotypes or phenotypes that do not have a plate screen. We report a novel method to monitor such phenotypes, where the fitness of mutants is assessed in mixed cultures under competitive growth conditions, and the abundance of any individual mutant in the pool is followed by means of its unique feature, namely the mutation itself. A mixed population of yeast mutants, obtained through transposon mutagenesis, was subjected to selection. The DNA regions (targets) flanking the transposon, until nearby restriction sites, are then quantitatively amplified by means of a ligation-mediated PCR method, using transposon-specific and adapter-specific primers. The amplified PCR products correspond to mutated regions of the genome and serve as 'mutant DNA fingerprints' that can be displayed on a sequencing gel. The relative intensity of the amplified DNA fragments before and after selection match with the relative abundance of corresponding mutants, thereby revealing the fate of the mutants during selection. Using this method we demonstrate that *UBI4*, *YDJ1* and *HSP26* are essential for stress tolerance of yeast during ethanol production. We anticipate that this method will be useful for functional analysis of genes of any microbe amenable to insertional mutagenesis.

INTRODUCTION

Complete sequencing of several microbial genomes has revealed thousands of genes of unknown function. Most of these genes seem to confer only subtle quantitative phenotypes (1–4) or play a role under conditions that cannot be reproduced on plate screens and thus cannot be readily identified. Though methods are available to monitor such phenotypes (5–8), they have a few limitations. Three of the methods (5–7) involve prior introduction of unique sequence tags in the mutants, which makes them laborious and costly. The other method, termed 'genetic footprinting' (8), does not involve sequence tags, but needs gene-specific primers and a PCR reaction for each gene to be monitored, which makes this method also costly and labor intensive.

Here we report a novel method to study the fitness of mutants in mixed populations. Unlike the other methods (5–8), this method does not need sequence tags or gene-specific primers to monitor individual mutants in the pool. Instead, mutated genes are tagged with transposon (Tn) insertions and the DNA regions flanking the Tn insertions are selectively and quantitatively amplified by a ligation-mediated PCR method. Since the amplified DNA fragments correspond to the mutated genes, they can be used to uniquely identify the mutants in mixed populations. This method will be useful for functional analysis of genes of any microbe amenable to insertional mutagenesis, as it can be used to readily monitor the abundance of individual mutants under competitive growth conditions.

MATERIALS AND METHODS

Yeast strains and plasmids

Saccharomyces cerevisiae strains disrupted in various heat shock protein genes were made by shuttle mutagenesis of strain FY3 (MATa *ura3-52*) (9) with plasmids having various HSP genes disrupted with transposon mTn-3xHA/lacZ. The disrupted genes (clones) *DDR48* (V6G5), *HSP104* (V8D8), *HSP104* (V22A9), *HSP35* (V18D3), *SOD2* (V4D11), *SSA1* (V6A2), *SSA2* (V18E7), *SSA3* (V41F1), *SSA4* (V5E8), *SSB1* (V23F11), *SSB2* (V32E7), *SSA4* (V3B8), *SSB2* (V47A3), *TPS2* (V2C2), *UBI4* (V36G6) and *YDJ1* (V45B4) were generously provided by M. Snyder's group (10,11). The positions of Tn insertions can be found in the TRIPLES database (12). Two different disruptions in *HSP26* were made in our laboratory by mutagenesis with mTn-3xHA/lacZ (11), and a disruption in *HSP82* was PCR amplified from a disruption library (11) with primers specific for *HSP82*. These disruptions were introduced into strain FY3 by transformation, with the disruption plasmids linearized with *Not*I or with the PCR product (for *HSP82*).

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Reconstruction experiment

Ten yeast mutants were grown individually in YPD (1% yeast extract, 2% peptone and 2% glucose) at 30°C and then mixed together at equal proportions to obtain a pool of eight mutants [pool A, *SOD2* (V4D11), *SSA2* (V18E7), *SSA3* (V41F1), *SSA4* (V3B8), *SSA4* (V5E8), *TPS2* (V2C2), *UBI4* (V36G6) and *YDJ1* (V45B4)] and a pool of two mutants [pool B, *HSP104* (V22A9) and *HSP35* (V18D3)]. These two pools were then mixed such that all 10 mutants were present at the same abundance or where the abundance of two mutants from pool B, with respect to other mutants, was decreased 2-, 4-, 8- or 16-fold. The genomic DNA was isolated from all the pools immediately and processed to amplify the targets.

Growth and fermentation

Nineteen HSP mutants of yeast were grown independently in YPD at 30°C for 20 h and mixed together in equal proportions to obtain a pool of 19 mutants. To monitor growth, cells from this pool were inoculated into fresh YPD medium at a starting OD_{600} of 0.05 and incubated at 30 or 38°C. The growth of cultures was monitored by OD_{600} at regular intervals and re-inoculated into fresh YPD broths when they completed five cell divisions. This was repeated for a total of 20 divisions. Genomic DNA was isolated from a starting pool of mutants and from the cells recovered after 5, 10, 15 and 20 cell divisions, and used for target display. Another part of the pool of mutants was transferred to fermentation medium (1% yeast extract, 2% peptone and 25% glucose) at a starting cell density of 0.15 g cells (wet weight) per 30 ml broth and incubated at 30 or 38°C with shaking. After incubation for 36 h (38°C) or 96 h (30°C), cells from 10 ml of the broth were harvested and inoculated into 50 ml YPD medium for outgrowth at 30°C. The outgrown culture was used to inoculate the next round of fermentation. Genomic DNA was isolated from outgrown cultures obtained after one and two rounds of fermentation and used for target display.

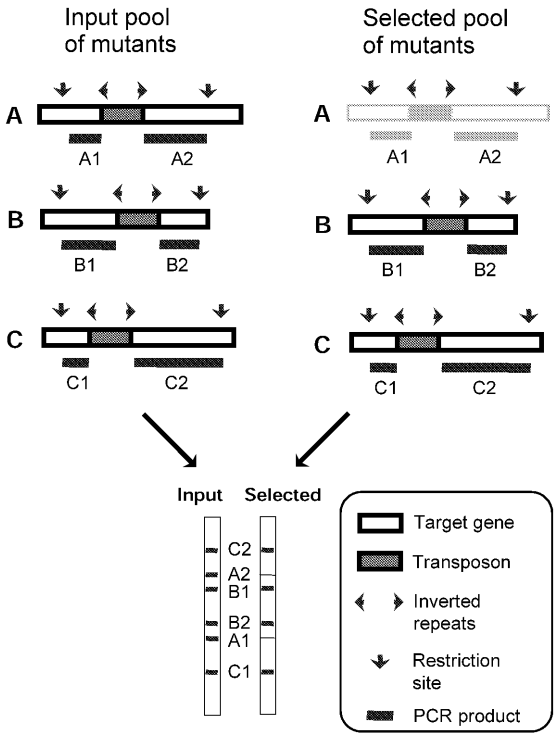

Figure 1. Schematic representation of Quantitative Target Display, a strategy to follow the abundance of mutants in mixed populations. The population of mutants is represented by the mutants A, B and C, mutated in the respective genes by the insertion of Tn. Mutant A is shown to have decreased in abundance during selection compared to the other mutants. The total genomic DNA of the input pool of mutants and that of the selected pool were purified and the DNA flanking the Tn insertion until nearby restriction sites (corresponding to mutated genes) were selectively and quantitatively amplified and resolved on a sequencing gel. The mutants that changed in abundance can be identified by comparing the intensities of PCR products of input and selected pools.

Quantitative target display

The genomic DNA isolated from individual or the mixed pool of mutants was digested with *Taq*I to completion (5 U/µg DNA for 4 h, in the reaction buffer and conditions suggested by New England Biolabs). The digest was diluted to 200 µl with 1× TE (pH 8.0) containing 100 mM NaCl and extracted with phenol–chloroform. DNA from the aqueous layer was precipitated at –70°C, for at least 30 min, with 2 vol of ethanol and 10 µg glycogen as a carrier. The pellet was washed with 70% ethanol, dried, and dissolved in sterile water. Purified DNA fragments were ligated to *Taq*I adapters (made by annealing oligos 5′-GACGATGAGTCCTGAG and 5′-CGCTCAGGACTCAT, both unphosphorylated; 13). A typical 10 µl ligation reaction contained 250 ng of DNA fragments, 5 µM *Taq*I adapters, 25 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM dithiothreitol, 500 µM ATP, 12.5 µg/ml BSA and 200 cohesive-end units of T4 DNA ligase (New England Biolabs) and was incubated at 16°C for 12–16 h. Primary PCR was done in a 25 µl reaction volume containing 200 µM each dNTP, 40 nM each primer (Taq1-N 5′-ATGAGTCCTGACCGA, and Tn3-O2, 5′-TTA-ACGTGAGTTTTCGTTCCACTG), 2 mM $MgSO_4$ and 1 U *Taq* DNA polymerase (Promega) in 1× PCR buffer (20 mM Tris–HCl pH 9.2, 10 mM KCl, 10 mM ammonium sulfate, 0.1% Triton X-100) and adapter-ligated DNA. The amount of adapter-ligated DNA used was 2.5 ng for individual mutants or 250 ng for pooled mutants, added directly from undiluted ligation reaction or from ligation reaction diluted 10-fold in water. For 'hot start', 15 µl of the reaction mixture in 1× PCR buffer having dNTPs, template, primers and $MgSO_4$ was overlaid with a 25 µl equivalent wax bead and incubated in the thermal cycler at 94°C for 30 s. The sample was then cooled to allow the wax to form a solid layer on top of the reaction mixture. Ten microliters of the enzyme mixture, having 1 U *Taq* DNA polymerase in 1× PCR buffer, was made separately and overlaid on the wax layer. The samples were kept in the thermal cycler (MJ Research Minicycler or Techne Genius) and taken through 30 cycles as follows. In all cycles the denaturation was done at 94°C for 30 s and extension at 72°C for 1 min, except for the additional 5 min extension after the last cycle. However, the annealing temperature, which to begin with was at 65°C for 30 s, was reduced by 0.7°C every cycle for 11 cycles, after which it was kept constant at 56°C. Semi-nested PCR was performed in a 25 µl reaction volume containing 1 or 2 µl of 10-fold diluted primary PCR product, 200 µM each dNTP, 200 nM each primers Taq1-N and Tn3-O1 (5′-GTTC-

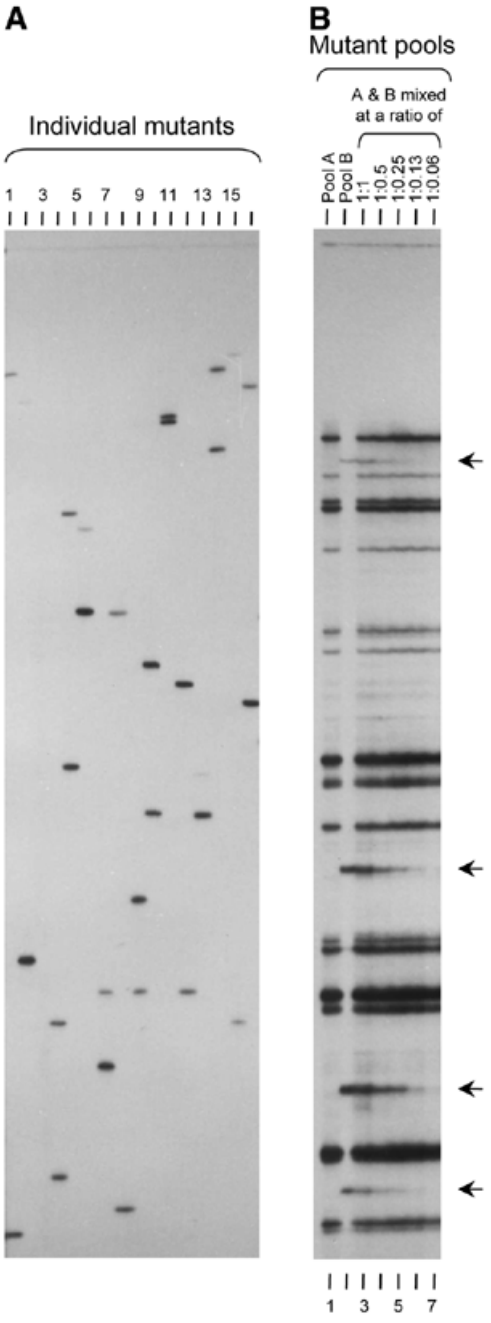

Figure 2. Selective and quantitative amplification of targets. (**A**) Selective amplification of DNA flanking Tn insertions. Genomic DNA of 16 individual mutants were used to amplify DNA fragments until nearby *Taq*I restriction sites, and resolved on a sequencing gel. The mutants analyzed were: lane 1, *SSA1* (V45B4); lane 2, *YDJ1* (V6A2); lane 3, *DDR48* (V6G5); lane 4, *SSA2* (V18E7); lane 5, *SSA3* (V41F1); lane 6, *SSA4* (V5E8); lane 7, *SSB1* (V23F11); lane 8, *SSB2* (V32E7); lane 9, *HSP35* (V18D3); lane 10, *SSA4* (V3B8); lane 11, *SOD2* (V4D11); lane 12, *SSB2* (V47A3); lane 13, *UBI4* (V36G6); lane 14, *TPS2* (V2C2); lane 15, *HSP104* (V8D8); lane 16, *HSP104* (V22A9). Two bands each are seen for most of the mutants, consistent with specific amplification of DNA from both sides of each Tn insertion. (**B**) Quantitative amplification of targets, demonstrated by a reconstruction experiment. Ten different Tn insertion mutants were grown individually and then mixed together in equal proportions to obtain a pool of eight mutants (pool A, lane 1) and two mutants (pool B, lane 2). These two pools were then mixed at different ratios such that the abundance of the two mutants from pool B, with respect to the other mutants, was the same (lane 3) or was decreased 2-fold (lane 4), 4-fold (lane 5), 8-fold (lane 6) or 16-fold (lane 7). Genomic DNA was isolated from all the pools immediately and processed to amplify the targets. Equal volumes of PCR products were loaded, except for lane 2 where it was one-fifth of other lanes. While the intensity of the bands from eight mutants remained constant in lanes 3–7, that of two mutants (arrows) decreased in proportion to the abundance of the mutants in the pools, confirming quantitative amplification of the targets.

CACTGAGCGTCAGACCC), 2 mM $MgSO_4$ and 1 U *Taq* DNA polymerase in 1× PCR buffer. Primer Tn3-O1 was end-labeled with [γ-^{32}P]ATP and polynucleotide kinase before being used. The thermal cycling was done as for primary PCR, except for the number of cycles, which was 35 in the semi-nested PCR. After PCR the reactions were mixed with an equal volume of 2× stop solution (95% formamide, 20 mM EDTA pH 8.0, 0.05% bromophenol blue and 0.05% xylene cyanol). The samples of individual mutants were further diluted 20-fold in 1× stop solution. Five microliters of 20-fold diluted or undiluted samples were heated at 72°C for 10 min, cooled on ice, resolved on a 5% denaturing acrylamide gel (33.0 × 39.5 × 0.05 cm) and autoradiographed.

RESULTS AND DISCUSSION

To identify genes conferring subtle and quantitative phenotypes or phenotypes without a plate screen, we have developed a novel method by which mutants are monitored in mixed populations by the features of the mutated genes themselves (Fig. 1). Here, transposon (Tn) mutagenesis is used to mutate and tag the genes and the DNA flanking the Tn insertions ('targets' of Tn insertions corresponding to mutated genes) are selectively amplified by PCR, using a primer specific for the terminal inverted repeats of the Tn and an adapter-specific primer. Two PCR products are expected for each mutant, one for each side of the Tn insertion. By comparing the intensity of the DNA fragments obtained from the mutant population before and after selection, it is possible to follow the fate of the mutants during selection.

The first requirement of the method is that it should be specific, i.e. only the DNA flanking Tn insertions, corresponding to the mutated genes, should be amplified. For this purpose, we have used a ligation-mediated PCR method reported for selective amplification of subsets of random restriction DNA fragments (AFLP) (13). Instead of random restriction fragments, our method is designed to amplify only the DNA flanking Tn insertions. The genomic DNA isolated from the mutants is digested with *Taq*I and ligated to an adapter compatible with *Taq*I overhang. As neither of the oligos of the adapter is phosphorylated, only one of the adapter oligos actually gets ligated to the ends of restriction fragments (through the 5′-phosphate of the restriction fragments). Unlike in AFLP, we employ a denaturation step prior to the addition of *Taq* DNA polymerase, to prevent synthesis of the sequence complementary to the ligated-adapter oligo. As the adapter primer used in PCR is of the same sense as the adapter oligo ligated to the ends of restriction fragments, it cannot anneal and initiate DNA synthesis unless the complementary strand is first made, which will occur only if there is a binding site for the Tn-specific primer. This ensures that only those DNA fragments adjoining Tn insertions are amplified. Since the Tn-specific primers

used correspond to the inverted repeats present at the termini of the Tn, two PCR fragments are expected for each mutant, one for each side of the Tn insertion. As seen in Figure 2A, lanes 1–16, individual mutants carrying single Tn insertions at different locations in their genomes yield at most two amplified DNA fragments, consistent with specific amplification of the targets. For some mutants only one fragment is seen; in these cases the other fragment is too large to be amplified and resolved by this method. Since the exact location of the Tn insertions in the genome is known for all the mutants (10,12), the size of the amplified DNA fragments could be predicted. Using a sequencing ladder as a size standard, the sizes of amplified fragments (up to 350 bases) could be experimentally determined (results not shown). These values matched with the predicted values, confirming the specificity of the method.

Another requirement of the method is that it should be quantitative, i.e. the intensity of the amplified DNA fragments should reflect the abundance of the respective mutants. This was tested by doing a reconstruction experiment, where the mutants were mixed in known proportions and subjected to quantitative target display. In a pool of 10 mutants, eight were kept constant and two were reduced. Only the four DNA fragments corresponding to these two mutants decrease in intensity in proportion to their abundance, confirming the quantitative nature of the method (Fig. 2B).

To test if this method can be used to trace the abundance of mutants during selection, a pool of mutants impaired in various heat shock protein genes was subjected to one or two rounds of ethanolic fermentation. This selection condition is not reducible to plate screens and, thus, as yet there is no direct screen to identify genes involved in stress tolerance during fermentation. The mutants were subjected to two rounds of fermentation at 30 or 38°C and analyzed by quantitative target display. To identify the mutants that could be impaired in growth, they were also grown up to 20 generations and analyzed (Fig. 3). The bands could be assigned to different mutants by comparing with the bands of individual mutants run in the same gel. Occasionally bands from different mutants overlap (e.g. *HSP104* and *SSA2*). However, as there are often two fragments amplified for each of these mutants, their fate during selection could be deduced from the non-overlapping bands. This problem can also be partially alleviated by using a different restriction enzyme for initial digestion of the genomic DNA, since the fragments from the same set of mutants are unlikely to overlap with the other enzyme as well. Still, overlap problems may become frequent with more complex pools of mutants, making it difficult to assign the identity of mutants with confidence. We envisage further developments of this method, based on differential hybridization of amplified targets to colony blots or DNA microarrays, to facilitate simultaneous monitoring of the abundance of hundreds of mutants in one pool.

With the exception of *HSP35*, all the mutants grew normally at both temperatures (Fig. 3). Several mutants decreased in abundance during fermentation, indicating that the corresponding wild-type genes are necessary for good survival during ethanol production. Among these, the role of *HSP104* in thermotolerance and ethanol tolerance is already known (14,15). On the other hand, this is the first study where a role in stress tolerance during ethanolic fermentation has been demonstrated for *UBI4*, *YDJ1* and *HSP26*. A role for *HSP26* is particularly striking as mutants of this gene have been very extensively studied to discover a biological role (16,17), but without any success. Thus, to further confirm the role of *HSP26*, an independent *HSP26* mutant was made by deleting this gene using a marker gene conferring G418 resistance (18). This mutant was then competed with the wild-type strain in mixed

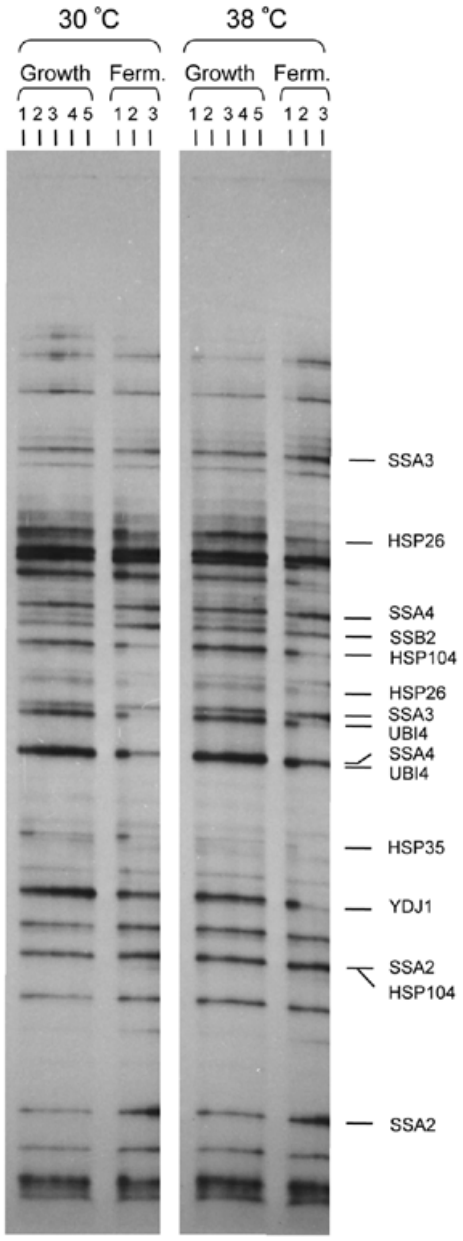

Figure 3. Relative abundance of mutants during growth and ethanolic fermentation. A mixed population of mutants singly mutated in various HSP genes by Tn insertions were monitored for their abundance during growth and fermentation at 30 and 38°C. To check the role of these genes in growth, targets were amplified from the starting population of cells (lane 1) or cells harvested after 5, 10, 15 and 20 generations of growth (lanes 2–5). To study the role of these genes during fermentation, targets were amplified from the starting population (lane 1) or cells harvested after 1 (lane 2) or 2 (lane 3) consecutive rounds of fermentation. Only the bands that changed in abundance are marked. They were assigned to different mutants by matching with the bands of individual mutants run in the same gel (not shown).

fermentation at 38°C and was found to decrease in abundance (results not shown), confirming the results obtained through quantitative target display. In contrast to the mutants that decrease in abundance, *SSA2*, and to some extent *SSA3*, paradoxically increase in abundance during fermentation (Fig. 3). The HSP70 proteins encoded by the *SSA* family of genes are thought to negatively regulate the level of HSPs (19,20); disruption of *SSA2* or *SSA3* presumably increases the level of HSPs, thereby resulting in increased survival during fermentation.

In conclusion, the quantitative target display method facilitates rapid discovery of the biological role of genes, since by using this method mixed culture competition experiments can be readily set up among Tn insertion mutants. For example, large collections (10,12) of such mutants available for yeast can be used for this purpose. The major advantage of this method over others (5–8) is that it does not need prior introduction of sequence tags (5–7) or gene-specific primers (8). Besides, as there is no need for prior genome sequence information to use this method, it can be easily applied to poorly studied pathogenic microbes of plants and animals, to facilitate a better understanding of their biology and pathogenicity.

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