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Development of a Whole-genome DNA Microarray for the Methylotrophic Yeast *Hansenula polymorpha*: Its Application to Transcriptome Profiling of Carbon Metabolism

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1. Introduction

Recent advances of DNA microarray technology provide a way to explore the metabolic and genetic control of genome-wide gene expression. The DNA array techniques have been most comprehensive in the traditional yeast *Saccharomyces cerevisiae,* which is the first eukaryotic organism with the whole genome completely sequenced (1). Analysis of gene expression patterns of *S. cerevisiae* using DNA microarray has contributed to understanding of the physiological aspects of cells, such as cell division and stress response to various environmental conditions. Apart from basic science, genome-wide expression analyses have been applied to obtain valuable information on the metabolic pathways in the industrial *S. cerevisiae* strains under several biotechnology processes, such as winemaking (2). DNA microarray technology is now employed to study other yeast species such as the fission yeast *Schizosaccharomyces cerevisiae* and the pathogenic yeast *Candida albicans*. Whole-genome DNA microarrays of *S. pombe* (3) and a partial genome array of *C. albicans* (4) have been developed and used for genome expression profiling studies.

The thermotolerant methylotrophic yeast *Hansenula polymorpha* has gained increasing interest as a useful system for fundamental research and applied purpose. *H. polymorpha* has been a favorable model to study the genetic control mechanism of methanol metabolism and peroxisome biogenesis. It has recently become one of the promising microbial hosts for the production of recombinant proteins on an industrial scale (5). Moreover, peculiar physiological characteristics of *H. polymorpha*, such as resistance to heavy metals, oxidative stress, and heat, make this yeast attractive for several biotechnological purposes (6). Successful exploitation of the industrial potential of *H. polymorpha* requires solid knowledge of the cellular systems at the global level.

2. Development of *H. polymorpha* cDNA microarrays: optimization and evaluation for transcript profiling

In an effort to obtain comprehensive information on gene function and regulatory networks in this yeast,

we have developed cDNA microarrays of *H. polymorpha*. As an initial approach, we had fabricated a partial genomic cDNA microarray spotted with 382 *H. polymorpha* ORFs and optimized procedures for target labeling and hybridization (7). At the time we had initiated our work on the development of the *H. polymorpha* DNA microarray, only partial sequence information on about 2,500 predicted open reading frames (ORFs) of *H. polymorpha* were publicly available as random sequenced tags (8). Each *H. polymorpha* ORF was PCR-amplified using gene-specific primer sets, of which the forward primers have 5'-aminolink. The PCR products were printed in duplicate onto the aldehyde-coated slide glasses to link only the coding strands to the surface of the slide via covalent coupling between amine and aldehyde groups. This generated single-stranded array elements minimizing the interference of complementary strands during hybridization (http://www.kribb.re.kr/metabolic/). Following completion of *H. polymorpha* whole genome sequencing (9), we have constructed *H. polymorpha* whole genome microarrays using the same strategy applied to the construction of the partial genome cDNA microarray. Based on information from the manual annotation of *H. polymorpha* complete genome sequence, which predicted 5, 848 ORFs, gene-specific PCR primers were designed using eprimer3 computer program to amplify the *H. polymorpha* whole ORFs.

Owing to its high-throughput nature, cDNA microarray technology is vulnerable to several systematic variations introduced during experimental processes. Thus, it is important to set up standard procedures for labeling of cDNA targets, prehybridization, hybridization, and slide washing to consistently generate high intensity and low background images. In an effort to optimize the conditions of analysis using our *H. polymorpha* genome cDNA microarray, we have compared the efficiency of two different cDNA labeling methods by self-against self hybridization. cDNA targets were prepared from total RNA isolated from the *H. polymorpha* cells cultivated in YPD medium, labeled with both Cy3 and Cy5, and then hybridized on the same array. We observed that the 3DNA indirect labeling method using fluorescent-labeled dendrimers exhibited a higher hybridization signal to noise ratio than the direct-labeling method, in which fluorescent nucleotides are directly incorporated into the cDNA targets. 3DNA labeling procedure consistently showed R-square values of 0.93 or higher and tight scatter plots, especially even for spots with low hybridization signals. Furthermore, the 3DNA indirect method generated reliable hybridization signals with much lower amount of starting RNA (about 25 μg RNA vs. 100 μg RNA) compared to the direct labeling method.

3. Application of *H. polymorpha* whole-genome DNA microarray to gene expression profiling of carbon metabolism

The whole-genome microarrays were used to investigate systematically the catabolite regulation of carbon metabolism in *H. polymorpha*. As methylotrophic yeast, it can grow on methanol, a cheap and pure substance, as the carbon and energy source. The metabolism of methanol in methylotrophic yeasts is induced by methanol but subject to strong repression by glucose, which is the primary and preferred fuel for eukaryotic microorganisms. We analyzed the temporal change of gene expression accompanying the carbon source shift from glucose to methanol and observed that genes involved in methanol metabolism

and peroxisome biogenesis were highly induced within 2 hours after shift to methanol in *H. polymorpha*, supporting prior knowledge on methylotrophic yeasts. Interestingly, the expression profiles also showed that several genes involved in glyoxylate cycle and pentose-phosphate pathway were significantly induced, whereas quite a few genes encoding the enzymes of the tricarboxylic acid (TCA) cycle were decreased in the cells cultivated on methanol. This finding strongly supports the notion that during methylotrophic growth the main source of NADH generation is not the TCA cycle but the oxidation pathway of methanol to CO_2 via formate.

We have also analyzed the gene expression profiles during glucose utilization and compared it to the temporal transciptome profiles accompanying the metabolic shift from fermentation to respiration in *Saccharomyces cerevisiae* (10). It was observed that in *H. polymorpha* many genes involved in TCA cycle were not repressed in glucose-rich medium, unlike the case in *S. cerevisiae*, indicating that respiration in *H. polymorpha* is repressed only partially in the presence of high concentrations of glucose. It was also noteworthy that many methanol-inducible genes, especially genes encoding enzymes involved in methanol utilization pathway, were highly expressed as glucose was depleted from the growth medium, demonstrating that the main regulation mechanism of the methanol metabolism is a depression/repression rather than an induction/repression by carbon sources. These findings provide invaluable information on the metabolic fate of glucose and methanol in the methylotrophic yeast *H. polymorpha*, which will be usefully applied for pathway engineering and process optimization in exploiting this yeast as a cell factory.

The changes of gene expression levels observed by cDNA microarray analysis were validated by semi-quantitative RT-PCR analysis. There was in general a close correlation between the microarray data and the RT-PCR analysis, supporting the validation of the results of cDNA microarray. On the other hand, we also confirm the expression pattern by evaluating the regulated expression of the reporter genes coding for *Aspergillus oryzae* glucose oxidase and a green fluorescent protein, respectively, fused to the promoters derived from noble inducible genes. The availability of the *H. polymorpha* DNA microarrays surely facilitates extensive exploration of interesting genes and strong promoters and will thus further development of expression systems to supplement the strong platform that already exists for *H. polymorpha*.

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