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Time-Course Data Analysis of Gene Expression Profiles Reveals *purR* Regulon Concerns in Organic Solvent Tolerance in *Escherichia coli*

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A time-course gene-expression profile was generated for *Escherichia coli* TK31 when it was exposed to an organic solvent mixture, and classified by fuzzy adaptive resonance theory (Fuzzy ART). It was found that the *purR* regulon plays an important role in the organic solvent tolerance (OST) of *E. coli*.

[**Key words:** *Escherichia coli*, organic solvent tolerance, *mar-sox* regulon, DNA microarray, fuzzy adaptive resonance theory (Fuzzy ART), *purR* regulon]

In recent years, there has been increasing interest in culturing microorganisms in two-phase systems that consist of an aqueous medium and a hydrophobic organic solvent. Although two-phase culture systems seem to be appropriate for the bioconversion of a substrate that has low solubility in water, organic solvents are genetically toxic to microorganisms (1, 2). The biological mechanisms of organic solvent tolerance (OST) in *Escherichia coli* have been investigated. The *mar-sox* regulon is widely known to be related to OST. It has been reported that MarA, a transcriptional activator of the *mar-sox* regulon, induced the expression of *acrAB* and *tolC* encoding the efflux pump thus contributing to the solvent resistance of *E. coli* (3–6). In addition, it has been also reported that some genetic determinants seem to give *E. coli* additional resistance to solvents (7–10).

We have studied OST mechanisms of *E. coli* using a DNA microarray (10, 11). Many genes were induced 30 min after exposure to organic solvents and one of the up-regulated genes, *glpC*, increased the OST of *E. coli* (10). Therefore, it was expected that the expression of OST-related genes in *E. coli* would dynamically change in the period 30 min after exposure to organic solvents. In the present study, to clarify the expression pattern of the genes induced by organic solvents and to discover new OST-related genes, the gene expression profiles of *E. coli* TK31 (10) at 0, 1, 2, 5, 10 and 30 min after exposure to mixed organic solvents of cyclohexane and *p*-xylene (6 : 4, v/v) were examined.

DNA microarray analysis was done as described previously (10). Briefly, TK31, a spontaneous *p*-xylene-tolerant mutant derived from JA300 (12), was cultured in LBGMg medium (13) at 37°C. For the control sample of DNA microarray experiments, 4 ml of the culture suspension were sampled when the cell turbidity, OD_{660}, reached 1.0. For the organic solvents-treated samples, we added 10% (v/v) of the organic solvents and then sampled 4 ml of the culture suspension. We regarded the suspension sampled at 5 s after solvent addition as the 0 min sample. The sampling experiments were done three times and total RNA was extracted from each sample. To investigate the gene expression, we used the IntelliGene™ *E. coli* CHIP ver. 2.0 (Takara Shuzo, Kyoto) and it was scanned using the GMS™418 Array Scanner (Genetic MicroSystems, Woburn, MA, USA). The IntelliGene™ *E. coli* CHIP ver. 2.0 contains 4155 immobilized DNA fragments corresponding to approximately 97% of all predicted open reading frames (ORFs) in *E. coli* K-12 W3110. Scanned images were analyzed by GenePix 3.0 (Axon Instruments, Union City, CA, USA). The fluorescence intensity of each spot was determined automatically and all spots were normalized so that the median of all spot ratios (Cy5/Cy3) would be 1.0.

In the present paper, we applied fuzzy adaptive resonance theory (Fuzzy ART) to analyze time-course data of gene expression profiles. ART is a type of self-organized clustering, which clusters a given set of input patterns into some groups. One of the characteristics of Fuzzy ART is the use of a similarity parameter, which is called the vigilance parameter ρ, and then the resulting number of clustered groups depends only on the similarity between all input patterns. Fuzzy ART has been developed for data analysis of DNA microarrays in our laboratory (14). Cluster construction obtained by Fuzzy ART showed high reproducibility and the highest clustering robustness was obtained even when adding random noise corresponding to 2-fold change. It was considered that Fuzzy ART can select genes with a similar

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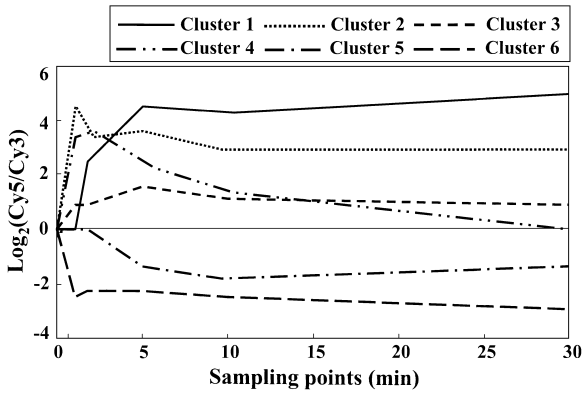

FIG. 1. Expression patterns analyzed by Fuzzy ART clustering. For microarray analysis, control RNA and sample RNA were labeled with Cy3-dUTP or Cy5-dUTP. Ratios of signal intensities were plotted against time after organic solvent addition (0, 1, 2, 5, 10, 30 min). Representative expression patterns obtained from Fuzzy ART are indicated in each cluster. The number of genes included in each cluster is as follows: cluster 1, 4; cluster 2, 10; cluster 3, 264; cluster 4, 33; cluster 5, 354; cluster 6, 11.

expression pattern with high robustness and correctness compared with other clustering methods, such as k-means or SOMs (15). In our previous paper, the MMP-3 gene, as a gene related to heat shock stress of cancer cells, was newly screened after clustering 664 heat-response genes by Fuzzy ART (16).

To classify genes by Fuzzy ART, the acquired data was processed according to the method of Kato *et al*. (16). For example, the gene expression data of the spots which were labeled as flagged data with low quality were omitted. As a result, 676 genes out of 4155 genes were selected and classified into 6 clusters by Fuzzy ART (Fig. 1). A total of 311 genes were classified into high-expression clusters, clusters 1–4. The genes in cluster 1 and 2 were strongly expressed at 30 min after exposure to the organic solvents. However, the pattern of cluster 1 was apparently different from that of cluster 2. Interestingly, cluster 1 contained *pspA* and cluster 2 contained *glpC*. Because the overexpression of *pspA* and *glpC* increases OST (10, 17), it may be possible that there are various biological mechanisms for OST in *E. coli* and some OST-related genes may be present in clusters 1 and 2. A total of 365 genes were classified into low-expression clusters, cluster 5 and 6. The genes in cluster 6 were strongly repressed at 1 min after exposure to organic solvent, while the genes in cluster 5 were repressed gradually.

We focused particularly on cluster 6 consisting of 11 genes. These genes are listed in Table 1. According to the regulonDB Data Base (http://www.cifn.unam.mx/Computational_Genomics/regulondb/), 7 genes, among 11 genes in Table 1, were members of the *purR* regulon which includes the genes repressed by PurR, a purine nucleotide synthesis repressor. Most genes of the *purR* regulon function as the enzymes of nucleotide metabolism. It is possible that the *purR* regulon is related to OST in *E. coli*, because it may be directly involved with the cell growth of *E. coli*.

Therefore, we tried to elucidate the relationship of PurR to OST in *E. coli*. First, we determined whether overexpression of *purR* in JA300 would increase tolerance to organic solvents. The *purR* gene was amplified with primers 5′-CGGAATTCGGGAATGGCAACAATAAAAGATGTAGC-3′ and 5′-GGGGTACCGGTGATTAACGACGATAGTCGC-3′ from the genomic DNA of JA300. The primer sequence was designed to contain an *Eco*RI and *Kpn*I site, shown by underlines. The high-copy number plasmid, pBluescript SK(+) II (Stratagene, La Jolla, CA, USA), was used for cloning and introduced into JA300. Figure 2a shows the results of the OST assay according to the method established by Nakajima *et al*. (6). Approximately 10^6, 10^5 and 10^4 cells were contained in each spot (Fig. 2a, column 1–3) and JA300(ppurR) formed many colonies in column 3. On the other hand, strain JA300(pBS) as a control formed several colonies in column 3. It is likely that overexpression of *purR* increased the OST of JA300. On the contrary, it is possible that the inactivation of *purR* decreases the OST level.

TABLE 1. Some of the genes in cluster 6

Gene	Encoded protein
ptsA	Phosphoenolpyruvate protein phosphotransferase
purB[a]	Adenylosuccinate lyase
purC[a]	Phosphoribosylaminoimidazolesuccinocarboxamide synthase
purD[a]	Phosphoribosylamine glycine ligase
purE[a]	Phosphoribosylaminoimidazole carboxylase catalytic subunit
purF[a]	Amidophosphoribosyltransferase
purL[a]	Phosphoribosylformylglycinamidine synthase
purN[a]	Phosphoribosylglycinamide formyltransferase
ydcI	Hypothetical transcriptional regulator
yecJ	Hypothetical protein
yojH	Probable malate : quinone oxidoreductase

[a] Genes repressed by *purR*.

Then, we investigated the effects of inactivation of *purR* on OST in JA300 using the OST assay (Fig. 2b). The chromosomal *purR* in JA300 was inactivated according to the method reported by Datsenko and Wanner (18). JA300(Δ*purR*) exhibited decreased colony forming effi-

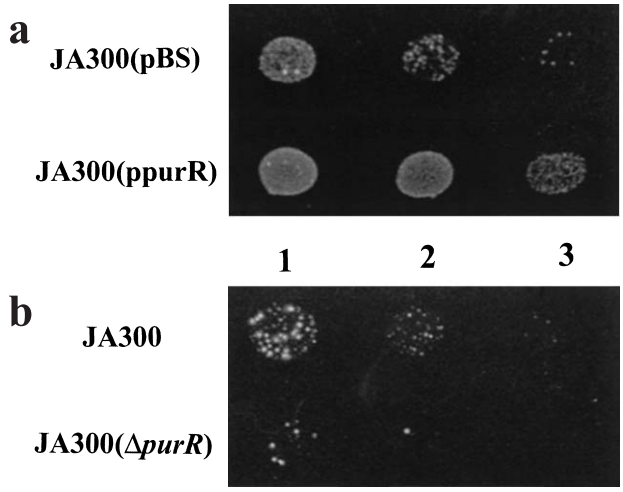

FIG. 2. (a) The effects of overexpression of *purR* in JA300 on OST. (b) The effects of inactivation of *purR* in JA300 on OST. The colony formation of *E. coli* on LBGMg agar overlaid with *n*-hexane for 24 h at 30°C. The spots contained approximately 10^6 (column 1), 10^5 (column 2), and 10^4 (column 3) cells.

ciency as predicted. Although the wild-type JA300 as a control exhibited several colonies in column 3, JA300(Δ*purR*) exhibited none. From the results in Fig. 2a and 2b, it was found that *purR* is one of the OST-related genes.

The regulonDB Data Base shows that PurR represses the expression of 26 genes. Among these 26 genes, 15 genes containing *purR* were not classified by Fuzzy ART because of deficient gene expression data. The other 11 genes were classified into low-expression clusters 5 and 6. In preliminary experiments, gene expression profiles were investigated when *E. coli* OST3410 (13) was exposed to *n*-decane or *n*-heptane. It was found that most of the 11 genes were down-regulated. Since *n*-decane and *n*-heptane are aliphatic compounds, we concluded that the effects of PurR described in the present paper were not specific effects of cyclic compounds, such as cyclohexane and *p*-xylene. Although the biological mechanisms of the down-regulation of the *purR* regulon by organic solvents are still unclear, it was assumed that the *purR* regulon may play an important role in the OST of *E. coli*.

In a previous study (10), we suggested that overexpression of genes encoding transcriptional regulator proteins using widely known information on the gene-expression network was one of the effective strategies for increasing the OST of *E. coli*. Although the *purR* regulon genes *purD* and *purT* (19) have not influenced the OST of *E. coli* by individual overexpression (10), PurR, a transcriptional activator of the *purR* regulon, influenced the OST of *E. coli* as expected. In addition, there were four genes, *ptsA*, *ydcI*, *yecJ* and *yojH*, in cluster 6. It has not been reported that these genes are members of the *purR* regulon and their expression is repressed by organic solvents. Further experiments about the role of these genes in OST are required.

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