**Note**

*soxRS* Gene Increased the Level of Organic Solvent Tolerance in *Escherichia coli*

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*Escherichia coli* strain JA300 grows in the presence of n-hexane, but not in the presence of cyclohexane. We isolated a 10.5-kb DNA fragment that provided cyclohexane tolerance on a multi-copy plasmid, from the chromosomal DNA of JA300. In this fragment, there were found C-terminal 10-amino acids truncated *soxR* (*soxR*) and *soxS* which control the superoxide response regulon genes. Characterization of subclones found that both the *soxR* and overexpression of the *soxS* increased the levels of organic solvent tolerance in several *E. coli* strains.

High concentrations of organic solvents are generally toxic to microorganisms. We have isolated and characterized a number of *Pseudomonas* strains from soils in Japan that were able to grow in high concentrations of toluene. From the physiological investigations of organic solvent tolerance of microorganisms, we use a parameter, log *P*sw, of organic solvents for the toxicity index of solvents. The log *P*sw is defined as the common logarithm of a partition coefficient (*P*sw) of a given solvent between n-octanol and water. An organic solvent the log *P*sw of which is the lower is the more toxic to microorganisms.

*Escherichia coli* strain JA300 (F−, leuB, trpC, thr, lac, thi, rpsL, hsdS) grows not only on LBGM agar (1% Bacto tryptone, 0.5% Bacto yeast extract, 0.5% NaCl, 0.1% glucose, and 0.25% MgSO4·7H2O, solidified with 1.5% agar) overlaid with n-hexane (log *P*sw 3.9) but in LBGM broth with n-hexane (10% v/v). But JA300 shows no growth in the presence of cyclohexane (log *P*sw 3.4). The organic solvent tolerance level is variable among *E. coli* strains. *E. coli* DH1 (supE, hsdR, recA, endA, gyrA, thi, relA) and W2252 (HfrC, metB, rel, thyA) showed no growth in the presence of n-hexane. The organic solvent tolerance in *E. coli* was found to be enhanced by mutations and one of the mutants grew in the presence of p-xylene (log *P*sw 3.1). Several genes should be involved in the organic solvent tolerance of *E. coli* strains. Genetic analysis between the JA300 and JA300-based n-hexane-sensitive strain OST4251 (same as JA300, but araD, ksgA, leu*, and n-hexane sensitive) identified the *ostA* gene, which decided n-hexane-tolerant phenotype. But the function of the *ostA* gene in organic solvent tolerance is still unknown. In this paper, to investigate the genes involved in organic solvent tolerance, we searched for genes that increased organic solvent tolerance in *E. coli*.

The chromosomal DNA of JA300 was partially digested by the restriction enzyme KpnI and ligated with Charomid 9-28 (Nippon Gene Co., Tokyo, Japan). Ligated DNA was packaged into phage particles and used to infect JA300 cells using the "Lambda Inn" in vitro Packaging Kit (Nippon Gene Co.). More

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Abbreviation: pBSII, pBluescript II.
than 3000 ampicillin resistant transfectants were spotted on LBGMg agar medium, overlaid with a 3-mm thick layer of cyclohexane and incubated at 30°C for 16 h. A plasmid pOST10 containing a 10.5-kb DNA insert was recovered from one of the cyclohexane tolerant transformants (Fig. 1). Subclones of the 10.5-kb fragment were constructed on a multi-copy vector (pBluescript II, Stratagene, LaJolla, CA, U.S.A.; abbreviated pBSII). One of the subclones containing a 1.5-kb KpnI–ClaI DNA fragment (designated pOST210) provided cyclohexane tolerance to JA300 (Fig. 1). The 1.5-kb DNA insert was mapped to the 92.2 min region of E. coli chromosome by Southern blotting analysis using the Escherichia coli Gene Mapping Membrane (Takara Shuzo Co., Kyoto, Japan). The DNA sequence and restriction enzyme map showed that the 1.5-kb DNA fragment included soxRS locus,10 lacking the C-terminal 10-amino acids of soxR gene (designated soxR). E. coli soxRS locus is organized as two head-to-head genes. The soxR and the soxS genes have overlapping transcripts and express SoxR (154 amino acids) and SoxS (107 amino acids) proteins, respectively.10 Analyzed DNA sequence of the 1.5-kb fragment was completely matched with the reported soxRS gene. We also cloned the complete soxRS gene on a 4-2 kb EcoR1–PvuII fragment (designated pOST221, Fig. 1) from the genomic library of JA300.11

E. coli soxRS genes control a superoxide response regulon.10 In the two-step transcriptional regulation model, constitutively expressed SoxR proteins are activated by superoxide anion radicals and increase the expression of soxS gene.12–14 The newly synthesized SoxS protein is the proximal activator of the soxRS regulon genes, such as sodA (manganese-containing superoxide dismutase), nfo (DNA repair enzyme endonuclease IV), zwf (glucose-6-phosphate dehydrogenase), fumC (redox resistant fumarase), and micF (OmpF posttranscriptional regulator).15–19

We constructed a variety of subclones containing soxRS, soxR, soxS, and soxS genes on the multi-copy vector and introduced them into the strain JA300. The JA300 cells harboring soxR gene (pOST220, pPOST210, pPOST220EV, and pPOST210EV) showed cyclohexane tolerance but not soxR (pPOST221, pPOST211, and pPOST211EV) (Fig. 1). We observed that E. coli cells carrying pPOST210EV (soxR) increased one of the soxRS regulon proteins, Mn-SOD (data not shown). This result coincided with the report that E. coli cells containing a C-terminal truncated soxR gene increased the expression of soxRS regulon genes.14 It was suggested that activation of soxRS regulon genes caused the cyclohexane tolerance.

The JA300 cells carrying the soxS gene under the control of a lac promoter on the multi-copy vector (pHeC3R) showed cyclohexane tolerance (Fig. 1). When the direction of the soxS insertion was opposite to the lac promoter on the vector (pHeC3), or when the insert was cloned on a low-copy vector (pPOST213), the JA300 transformants showed no growth in the presence of cyclohexane (Fig. 1). These results suggested that the cyclohexane tolerance was dependent on high-level expression of the soxS gene.

We investigated the effects of the soxRS gene on the organic solvent tolerance of various E. coli strains. When n-hexane sensitive strains DH1 and OSTE4251 were used, cells carrying pPOST210 (soxRS) or pHeC3R (soxS) showed the growth in the presence of n-hexane (Table). The OSTE4251 cells carrying pHeC3R grew

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(++) abundant growth, (+) poor growth, (−) no growth.

Abbreviations of organic solvents: DE, diphenylether; H, n-hexane; CH, cyclohexane; MixH/CH, mixed solvent of n-hexane and cyclohexane (1:1); pX, p-xylene. Freshly grown E. coli cells were suspended in 0.8% NaCl (approx. 10^7 cells/ml) and one drop of the suspension was spotted on solid LBGMg medium consisted of 1% Bacto tryptone (Difco Laboratories, Detroit, MI, U.S.A.), 0.5% Bacto yeast extract (Difco Labs.), 1% NaCl, 0.1% glucose, and 100 mm MgSO_4 (pH 7.0). The surface of the medium was overlaid with organic solvent of 3 mm thickness. The plates were incubated at 37°C for 16 h.

Fig. 2. Growth of E. coli JA300 Carrying soxRS Plasmid in the Presence of Cyclohexane.

E. coli JA300 cells carrying pPOST210 were incubated in 10 ml LBGMg medium at 37°C, and at the time indicated with arrows, 1 ml of cyclohexane was added and incubated at 37°C with shaking. (a) Growth of the cultures were monitored by measuring the absorbance at 660 nm. (b) The cultures were samples periodically and viable cells were counted on LBGMg plates. (c) JA300 (pPOST210), no organic solvent; (▲) JA300 (pBSII); (●) JA300 (pPOST210).
even in the presence of cyclohexane. The effect of pHc3R was stronger than that of pOST210 on organic solvent tolerance of both OST4251 and DH1 cells. *E. coli* MC1064 (*hsdR*, *araD, ΔaraABC-leu*), lacX, galU, galK, rpsI, thi, *n*-hexane tolerant10 also acquired cyclohexane tolerance with both the pOST210 and pHc3R. These results suggest that the multi-copy *soxS* gene under a strong promoter generally increases the organic solvent tolerance level of the host *E. coli* strains. However, we observed that *E. coli* OST3410 (same as JA300, but cyclohexane tolerant)50 carrying pPOST210 or pHc3R did not grow in the presence of p-xylene (Table) and a mixed solvent of cyclohexane and p-xylene (7:3, v/v) (data not shown).

JA300 cells with or without pPOST210 were grown in LBGMg medium, and cyclohexane was added at the early exponential phase of growth (OD_{660} 0.05, number of viable cells: 4 × 10^8/ml) to give a final concentration of 10% (v/v). Growth of JA300 cells was completely stopped by adding cyclohexane. The number of viable JA300 cells was radically decreased to 2 × 10^2/ml within 5 min after adding cyclohexane. On the other hand, the growth of the JA300 cells carrying pPOST210 continued in the presence of cyclohexane and the final OD_{660} reached 1.0. The number of viable cells of JA300 (pPOST210) was maintained at 10^6–10^7/ml (Fig. 2).

Activation of the *soxRS* gene by superoxide-generating reagents dramatically increases the expression of Mn-superoxide dismutase (Mn-SOD).15,16 We analyzed the cellular protein of *E. coli* cells exposed to organic solvents, but the induction of Mn-SOD was not observed (data not shown). This indicated that the exposure to organic solvents was not an activation factor for the *soxRS* regulon.

Activation of the *soxRS* system increases resistance to superoxide generating reagents and a number of antibiotics (chloramphenicol, nalidixic acid, tetracycline, etc.).18 It was suggested that the multi-drug defense responses induced by *soxRS* regulon caused the increase of organic solvent tolerance in *E. coli* cells.

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**References**