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REVIEW

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Improvement of organic solvent tolerance level of *Escherichia coli* by overexpression of stress-responsive genes

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Abstract Water-immiscible organic solvents can be toxic to microorganisms. The tolerance levels differ among strains of *Escherichia coli*, suggesting that the organic solvent tolerance level is strain specific and determined genetically. We constructed several mutants from *E. coli*, of which the organic solvent tolerance levels were improved. The mutants were defective in the *marR* gene encoding a repressor protein for the *mar* operon that is responsible for environmental stress factors. High expression of stress-responsive genes, *soxS, marA*, and *robA*, elevated organic solvent tolerance levels of several strains of *E. coli*. These genes code for DNA-binding proteins that are transcriptional activators belonging to the AraC subfamily with the helix-turn-helix motif. It was shown that expression of the AcrAB-TolC system, a major efflux pump in *E. coli*, was positively regulated by the proteins. This system was highly expressed in the organic solvent-tolerant mutants. Strains defective in one of the genes, *acrA*, *acrB*, or *tolC*, were remarkably sensitive to organic solvents.

Key words Organic solvent tolerance · *Escherichia coli* · Stress-responsive genes · *mar* operon · AcrAB · TolC

Introduction

Hydrophobic organic solvents can be toxic to organisms. Environments containing high concentrations of organic solvents represent one definition of extreme environments for organisms, such environments being found mainly in areas polluted with petroleum or synthetic organic solvents. Microorganisms are also harmed by various organic solvents. For example, chloroform and toluene, typical solvents conveniently used in many laboratories, are highly toxic. Toluene has been used to sterilize microbial cultures and to maintain solutions in a sterile condition. Toluene is also used as an unmasking agent in the assay of several intracellular enzymes, such as β-galactosidase. There are some microorganisms that can assimilate toxic organic solvents. However, concentrations of the solvents supplied for these microorganisms are low. In recent years, there has been an increasing interest in culturing microorganisms in two-liquid phase systems consisting of organic solvent and aqueous medium. This approach possibly provides a convenient means of bioconversion of hydrophobic organic compounds (de Smet et al. 1983; Harbron et al. 1986; Favre-Bulle et al. 1991; Van Sonsbeck et al. 1993; Aono et al. 1994a; Doukyu and Aono 1997).

There are microorganisms producing organic solvents, such as lactic acid, ethanol, glycerol, acetone, or butanol. These fermenters are found widely. In particular, ethanol has been widely fermented by brewers. The fermenters are killed by their own products accumulating to certain concentrations in media. However, highly organic solvent-tolerant microorganisms have been isolated (Inoue and Horikoshi 1989; Aono et al. 1992; Cruden et al. 1992; Nakajima et al. 1992; Weber et al. 1993; Ramos et al. 1995). These strains grow in the presence of a large volume of *p*-xylene or toluene, which are highly harmful to microorganisms.

We have studied microbial tolerance mechanisms to organic solvents using *Escherichia coli*. Organic solvent tolerance levels of *E. coli* can be improved by mutations or transformations of cloned genes conferring the organic solvent tolerance (Aono et al. 1991, 1994b, 1998; Nakajima et al. 1995a,b; Asako et al. 1997). These genes encode various proteins located in cytoplasm or the inner or outer membrane. This article summarizes an empirical rule concerning organic solvent toxicity to microorganisms and *E. coli* genes involved in determining organic solvent tolerance.

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Log P_{ow}, a scale of organic solvent toxicity to microorganisms

Most organic solvents are of low polarity and are not freely miscible with water. When a large volume of low polarity organic solvent is added to water, the resulting mixture forms two layers. The water layer is saturated with the organic solvent. Most microorganisms cannot grow in liquid or on solid media overlaid with highly toxic organic solvents such as benzene, toluene, and *p*-xylene. Organic solvent-tolerant microorganisms discussed here are defined as organisms that can grow in the presence of a large volume (10%–50% of volume of the medium) of low polarity organic solvents. We proposed that this biphasic system be termed "persolvent system" or "oleosus system" to emphasize the presence of an excess volume of organic solvent (Aono et al. 1994a,b).

Toxicity is highly variable among organic solvents, as is empirically well known. For instance, bacteria are sometimes grown in medium overlaid with liquid paraffin to avoid exposure to the external atmosphere. The paraffin does not suppress the growth of most bacteria. On the other hand, some organic solvents, such as chloroform, are strongly toxic and have been used to kill and lyse various bacterial cells. As described, excess volumes of various organic solvents overlay culture media. On agar media, bacterial cells are in direct contact with the solvent overlying the agar. In liquid media, bacteria grow in the aqueous phase saturated with the solvent. A concept of lethal dose, which has been measured by administration of a small amount of some compound to animals, cannot be applied for assessing toxicity of a particular organic solvent used in a such manner.

An empirical rule is proposed to evaluate the toxicity on colony development by various microorganisms on a complex medium overlaid with organic solvent. The colony development was most correlated with the log P_{ow} value among several physicochemical parameters of the organic solvents (Inoue and Horikoshi 1989; Aono et al. 1991). The log P_{ow}, the common logarithm of P_{ow} that is a partition coefficient of the organic solvent between *n*-octanol and water (Leo 1993), is inversely correlated with toxicity of the solvent (Table 1). An organic solvent with a lower log P_{ow} is more toxic to microorganisms. Each microbe can grow on the medium overlaid with a variety of organic solvents whose log P_{ow} values are equal to or greater than a particular value. This log P_{ow} value is called the index value for growth of each organism. A solvent whose log P_{ow} value is equal to the index value is called the index solvent (Aono et al. 1994a,b). The index value representing the tolerance level of each microorganism is different among genera, spe-

Table 1. Organic solvent tolerance (OST) levels found in several microorganisms

Solvent	log P_{ow}	*Pseudomonas putida* IH-2000	*Pseudomonas putida* Px51T	*Pseudomonas putida* IFO3738	*Pseudomonas fluorescens* IFO3507	*Escherichia coli* IFO3806	*Achromobacter delicatulus* LAM1433	*Alcaligenes faecalis* JCM1474	*Agrobacterium tumefaciens* IFO3058	*Bacillus subtilis* AHU1219	*Saccharomyces uvarum* ATCC26602
Dodecane	7.0	+	+	+	+	+	+	+	+	+	+
Decane	6.0	+	+	+	+	+	+	+	+	+	−
Nonane	5.5	+	+	+	+	+	+	+	+	+	−
n-Hexyl ether	5.1	+	+	+	+	+	+	+	+	+	−
Octane	4.9	+	+	+	+	+	+	+	+	+	−
Isooctane	4.8	+	+	+	+	+	+	+	+	−	−
Cyclooctane	4.5	+	+	+	+	+	+	+	−	−	−
Diphenyl ether	4.2	+	+	+	+	+	+	−	−	−	−
n-Hexane	3.9	+	+	+	+	+	+	−	−	−	−
Propylbenzene	3.7	+	+	+	+	+	−	−	−	−	−
o-Dichlorobenzene	3.5	+	+	+	+	−	−	−	−	−	−
Cyclohexane	3.4	+	+	+	+	−	−	−	−	−	−
Ethylbenzene	3.2	+	+	+	−	−	−	−	−	−	−
p-Xylene	3.1	+	+	+	−	−	−	−	−	−	−
Styrene	2.9	+	+	−	−	−	−	−	−	−	−
Toluene	2.6	+	+	−	−	−	−	−	−	−	−
Benzene	2.1	−	−	−	−	−	−	−	−	−	−
Index value		2.6	2.6	3.1	3.4	3.7	3.9	4.5	4.8	4.9	7.0

+, growth; −, no growth.
Results cited from Inoue and Horikoshi (1989).

cies, and strains of bacteria (Inoue and Horikoshi 1989, 1991; Aono et al. 1991). This toxicity concept of the lethal toxicity of organic solvents can be applied to most microorganisms so long as the organic solvent does not react chemically with some outermost constituents of microorganisms.

Toluene is highly toxic to microorganism, as represented by its log P_{ow} value. The first toluene-tolerant microorganism, *Pseudomonas putida* IH-2000, was isolated from soil taken from a grassland and reported in 1989 (Inoue and Horikoshi 1989). Isolation of this strain prompted a survey of toluene-tolerant microorganisms, and we collected a large number of toluene-tolerant microbes from soil samples (Aono et al. 1992; Nakajima et al. 1992), showing that such microorganisms are considerably widespread. These tolerant strains were isolated from soils of areas not polluted with any chemicals, indicating that the toluene tolerance had not been developed to adapt to chemical pollutants. The toluene-tolerant microbes isolated by us belong to the fluorescens group of *Pseudomonas* (*P. aeruginosa, P. fluorescens*, *P. putida*). Strains belonging to *P. putida* were predominant among them. Other tolerant strains reported also belong to *P. putida* (Cruden et al. 1992; Weber et al. 1993; Ramos et al. 1995).

Table 1 shows bactericidal toxicity but not bacteriostatic toxicity of organic solvents. Organic solvents seem to kill microbial cells by some common mechanism. Probably the bactericidal mechanism is based on perturbation of microbial membrane structures (Sikkema et al. 1994, 1995). It is known that hydrophobic compounds accumulate in phospholipid liposomes (Sikkema et al. 1992). The extent of this accumulation is positively correlated with magnitude of the log P_{ow} of the compound, meaning that organic solvents of low toxicity accumulate more abundantly in the liposomes than more toxic solvents. Therefore, the toxicity level of a particular organic solvent cannot be explained only by the accumulation tendency shown in the liposomes.

Organic solvents with lower log P_{ow} values bind more abundantly to viable cells of *E. coli* (Aono and Kobayashi 1997). This finding explains the basis of the empirical log P_{ow} toxicity rule and suggests that some structure or function of the outermost cell surface prevents binding of hydrophobic organic solvents. It is likely that effectiveness of some steps (adhesion of organic solvents with the cell surfaces, occlusion of organic solvents in the outermost strata, intercalation of organic solvents into the membranes, and export of the solvent from the membrane) responds to the polarity of the organic solvent.

Growth characteristics of *E. coli* in the persolvent system

The biochemical and genetic properties of the cell structures of *E. coli* are well understood. This knowledge is useful in understanding the organic solvent tolerance mechanisms. In contrast, the knowledge is poor in the case of *P. putida.* Although *E. coli* is less tolerant to organic solvents than *P. putida*, its tolerance level is relatively high among various microorganisms (Table 1). Organic solvent tolerance mechanisms could be clarified by using appropriate solvent-tolerant strains of *E. coli* K-12. Correlation between toxicity and log P_{ow} value of organic solvents is also found in *E. coli*.

Supplements of Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} ions enhance the organic solvent tolerance levels of *E. coli* (Aono et al. 1994b). These four alkaline earth cations show the maximum effects at 5–10 mM when *E. coli* JA300 is grown in LBG medium (1% Bacto tryptone, 0.5% yeast extract, 1% NaCl, 0.1% glucose) or on LBG agar medium. Effects of these ions are additive but not synergistic. The log P_{ow} toxicity correlation depends on the culture conditions. The role of the alkaline earth cations is probably stabilization of membrane structure by reduction of charge repulsion between anionic molecules in membranes.

Growth of *E. coli* in liquid medium is inhibited by organic solvents. The observed magnitude of the growth inhibition depends on the strain examined, log P_{ow} value of the organic solvent, and concentration of the alkaline cations (Aono et al. 1994b). Figure 1 shows the effet of sudden addition of various organic solvents on growth of JA300. Isooctane (log P_{ow} 4.8) did not at all inhibit growth of JA300 cells growing in LBG Mg (Fig. 1B). Cyclooctane (log P_{ow} 4.5) inhibited growth after the cells grew to high density. The cells became hypersensitive to the organic solvent after O_2 in the vessel was consumed (Noguchi et al. 1997). Microbial organic solvent tolerance is dependent on bioenergy, as described next. *n*-Hexane (log P_{ow} 3.9) slightly inhibited immediately after addition and killed after the cells were grown to high cell density. Cyclohexane (log P_{ow} 3.4) interrupted growth and caused a rapid decrease in the number of viable cells. Almost all cells were killed with only a few cells surviving. The survival frequency was 10^{-5} to 10^{-6}. Surviving cells grew in the presence of cyclohexane at a slower rate than that in the absence of cyclohexane. *p*-Xylene (log P_{ow} 3.1) rapidly and completely killed JA300 cells. The number of viable cells was less than 10/ml at 10 min after the addition. No viable cell was detected after 24 h. Turbidity of the cultures did not decrease after the addition of the organic solvents (Fig. 1B).

The lethal effect of *n*-hexane was significant on the cells growing in LBG medium (Fig. 2). The survival frequencies were 14% in LBGMg at 20 min after the addition and 6×10^{-5} in LBG, respectively. The culture not exposed to *n*-hexane contained *n*-hexane-tolerant cells at a similar frequency. After the addition of *n*-hexane, the culture was enriched in the tolerant cells by selection of the *n*-hexane-tolerant cells rather than adaptation of the *n*-hexane-sensitive cells to *n*-hexane. *n*-Hexane tolerance found in these cells was not stably heritable when the cells were grown without *n*-hexane.

Ultrastructural changes in *E. coli* grown in the presence of organic solvents

Strongly toxic solvents such as toluene are believed to break down microbial membranes. *n*-Octane intercalates in the

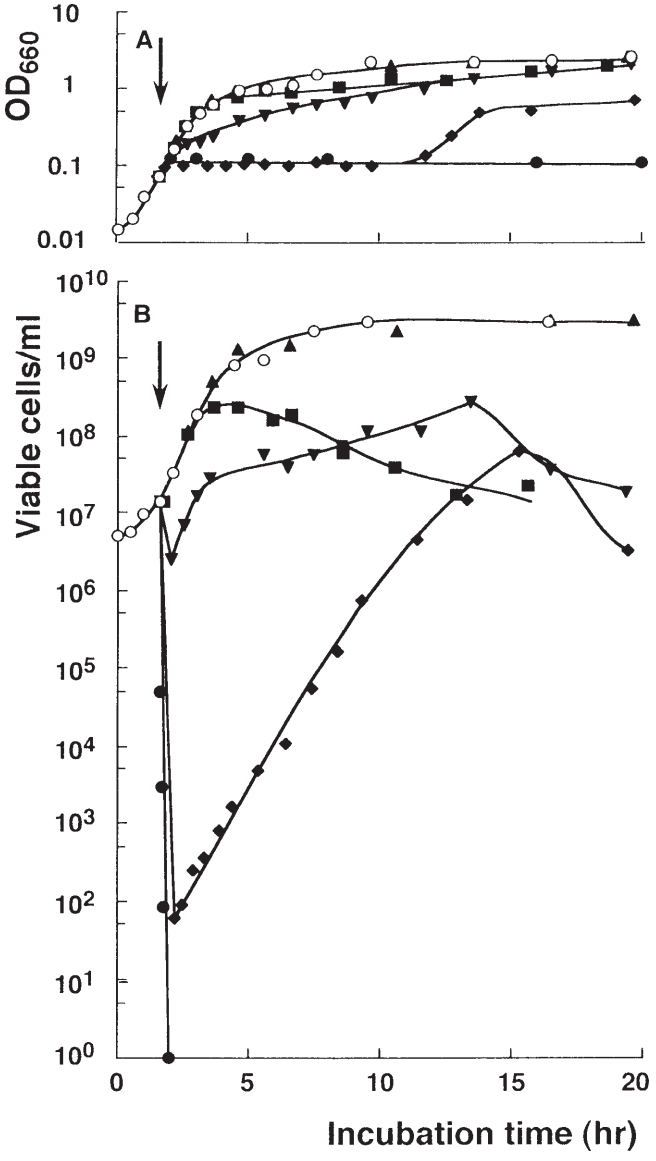


Fig. 1. Effects of organic solvents on growth of *E. coli* JA300 grown in LBG Mg medium at 37°C. At the time indicated with *arrows*, organic solvent (10% volume of the medium) was added to the culture: isooctane (*up-pointing triangles*), cyclooctane (*squares*), *n*-hexane (*down-pointing triangles*), cyclohexane (*diamonds*), and *p*-xylene (*solid circles*). As a control, no organic solvent was added (*open circles*). OD_{660} and the number of viable cells in the aqueous layer were determined; the number of viable cells was counted on LBG agar

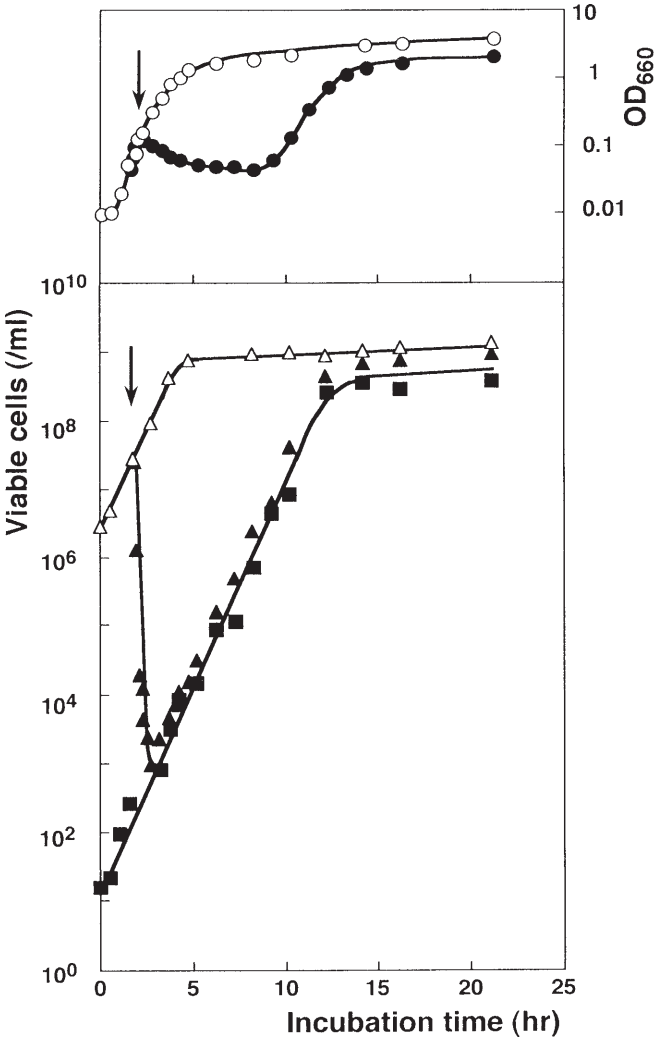
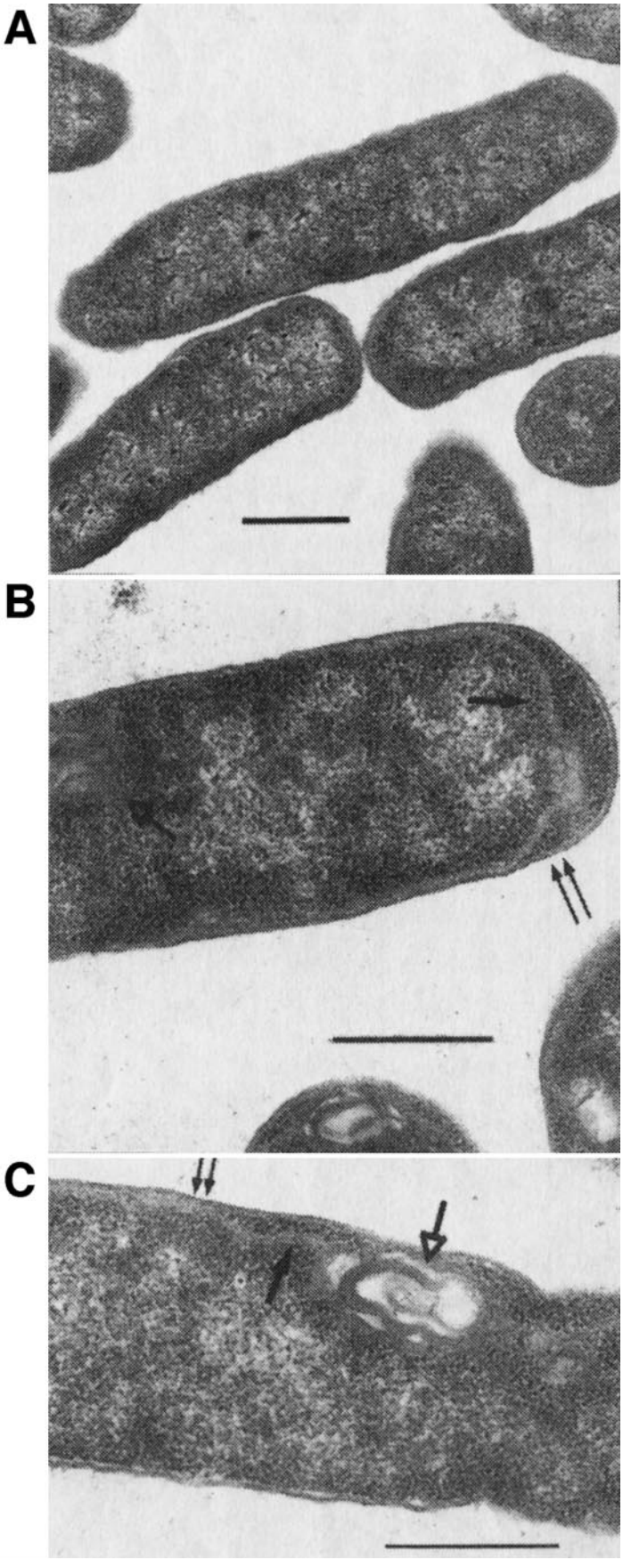


Fig. 2. Growth of *E. coli* JA300 in LBG medium overlaid with *n*-hexane. At the time indicated with *arrows*, *n*-hexane (10% volume of the medium) was added to the culture. OD_{660} (*circles*) and the number of viable cells in the aqueous layer were examined; the number of viable cells was counted on LBG agar overlaid (*squares*) or not overlaid (*triangles*) with *n*-hexane. *Solid symbols*, *n*-hexane was added; *open symbols*, *n*-hexane was not added

outer membrane of *E. coli* (Favre-Bulle et al. 1991). Phospholipid liposomes are swollen by intercalation of various organic solvent molecules (Sikkema et al. 1994). These results indicate that the primary site of growth inhibition with organic solvents is the biological membrane (Sikkema et al. 1995).

Organic solvents, such as *n*-hexane, cyclohexane, and *p*-xylene, have a marked effect on the inner membrane structure of *E. coli* JA300 (Aono et al. 1994b). The outer membrane of JA300 cells incubated in the presence of *n*-hexane is clearly shown by electron microscopic observation (Fig. 3B) because of displacement of the inner and outer membranes. An expanded periplasmic space was filled with ribosome-like particles, suggesting that the inner membrane is broken at least once by the solvent, allowing leakage of cytoplasmic ribosomes into the periplasm. The outer membrane appeared relatively not perturbed. The primary site of damage by organic solvents is likely the inner membrane. The damage might interfere in various metabolic activities that are carried out on the inner membrane, such as bioenergy production.

Large membranous invaginated bodies were observed in the cytoplasm of the cells treated with the solvent. These spherical structures consisted of tightly folded and coiled membranes, and were shown in transverse and longitudinal section (Fig. 3C). They were found mainly at the lateral faces of cells but sometimes occurred at the poles. In some



thin sections, the inner membrane could be seen directly connected to invaginated bodies. Occurrence of these bodies might suggest over-enhancement of lipid synthesis.

These observations imply that *E. coli* cells exposed to organic solvents are subject to strong stress. In fact, PspA (phage shock protein A), a peripheral inner membrane protein, was strongly induced in the cells grown in the presence of organic solvent (Kobayashi et al. 1998), as shown in Fig. 4. This protein is known to be induced in *E. coli* whose membrane structure is damaged under extreme stress conditions, such as heat shock, hyperosmotic shock, ethanol treatment, protein secretion inhibition, fatty acid synthesis inhibition, ATP synthesis inhibition, and prolonged incubation in the stationary phase of growth (Model et al. 1997).

Dependence of organic solvent tolerance on bioenergy production

In the presence of toluene, growth of a toluene-tolerant strain, *P. putida* Px51T that was isolated by us (Nakajima et al. 1992), was dependent on O_2 supply. The organic solvent tolerance level decreased greatly under microaerobic conditions. This is also true for *P. aeruginosa* and *E. coli* (Noguchi et al. 1997). The tolerance levels of *P. aeruginosa* and *E. coli* were restored in the presence of nitrate even under anaerobic conditions. The number of viable cells of Px51T decreased markedly on exposure to organic solvents with log P_{ow} value greater than the index value by pretreatment with a proton conductor, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), or an electron transport chain inhibitor, NaN_3. The organism was highly susceptible to organic solvents also when starved for a carbon source. The CCCP treatment also lowered the organic solvent tolerance of *E. coli*. These results indicate that some bioenergy is required for maintenance of the organic solvent tolerance, which can explain why organic solvent tolerance levels of microorganisms are not stable at high cell density (see Fig. 1).

The level of the ATP pool in the cells was not lowered by the treatments, and ATP synthetase inhibitors (aurovertin, *N,N′*-dicyclohexylcarbodiimide, and vanadate) showed no effect on solvent tolerance, at least for 30 min. Treatment with CCCP reduced the levels of organic solvent tolerance of *P. aeruginosa* and *E. coli* as well as of *P. putida*. These results suggest that the organic solvent tolerance levels of microorganisms may be dependent on the magnitude of the proton motive force. It is reported that the toluene-tolerant

Fig. 3A–C. Effects of *n*-hexane on the ultrastructure of *E. coli* JA300 grown in LBG medium at 37°C. At midexponential growth phase, the cells were incubated with *n*-hexane (10% v/v) for 3 h. **A** Without *n*-hexane; **B**, **C**, with *n*-hexane. After 3 h at 37°C, the cells were fixed with 4% (v/v) glutaraldehyde at pH 7.4; the cells were postfixed with OsO_4. Preparations were stained with uranyl acetate and counterstained with lead citrate. Outer membranes (*double arrows*); cytoplasmic membranes (*single arrows*); membranous invaginated bodies (*open arrow*). *Bars*, 0.5 μm (**A**, **B**); 0.25 μm (**C**)

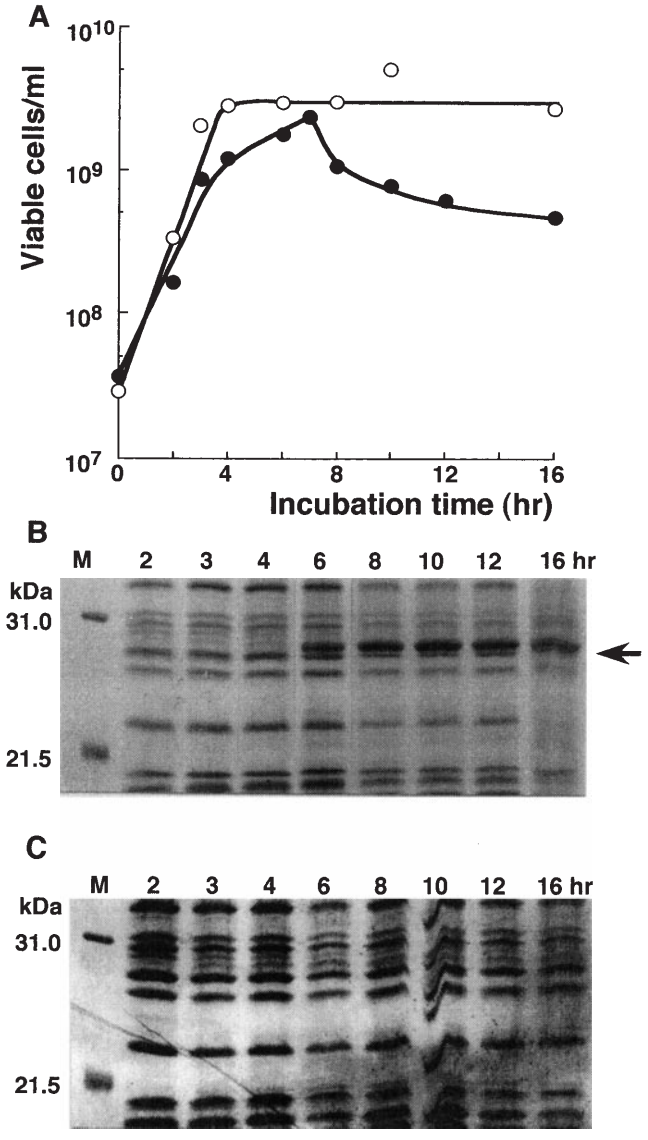


Fig. 4A–C. Appearance of phage shock protein A (PspA) in *E. coli* JA300 grown in LBG Mg medium overlaid with 10% (v/v) cyclooctane (*solid circles*) or without added solvent (*open circles*). **A** The number of viable cells in the medium layer was periodically examined. Cells were harvested from the culture with cyclooctane (**B**) or without added solvent (**C**) at the times indicated at the top of each gel. Sodium dodecylsarcosine (0.5%) -soluble protein obtained from the whole envelope containing 30 μg of protein was electrophoresed on an SDS-polyacrylamide gel. Protein was stained with Coomassie brilliant blue R-250. Protein PspA is indicated by an *arrow*. *M*, molecular mass markers

P. putida strain accumulates toluene abundantly in the cells by treatment with CCCP or KCN (Isken and de Bont 1996).

Improvement of organic solvent tolerance level of *E. coli* by stepwise mutations

Although organic solvent tolerance levels of individual microbes are influenced physiologically, the intrinsic levels are probably determined genetically. We constructed several organic solvent-tolerant (OST) mutants from *E. coli* JA300 (Aono et al. 1991). Spontaneous cyclohexane-tolerant mutants were first isolated from among derivatives that formed colonies on LBGMg agar medium overlaid with cyclohexane (Table 2). Derivatives with similar phenotypes are obtained from surviving cells grown in LBG Mg medium overlaid with cyclohexane (Fig. 1). Among the cyclohexane-tolerant clones, OST3408 and OST3410 were further analyzed. From OST3408 cells, a spontaneous *n*-pentane-tolerant mutant (OST3301) was selected. A *p*-xylene-tolerant mutant (OST3101) was isolated from OST3301. Mutant OST3101 grew on the agar medium overlaid with *p*-xylene but showed poor growth in the liquid medium containing a high concentration of *p*-xylene. A mutant (OST3121) that grew in the medium containing 10% (v/v) *p*-xylene was isolated from OST3101 after treatment with a mutagen, NTG.

The OST mutants have also acquired tolerance toward low levels of hydrophobic antibiotics, such as ampicillin, chloramphenicol, and tetracycline (Aono et al. 1995). These results suggest that OST mutants might differ from the parent in characteristics of the cell surface. Microbial adhesion to hydrocarbon tests (Rosenberg and Doyle 1990) showed that the cell-surface hydrophobicity of each OST mutant is lower than that of the parent (Table 3). The magnitude of decrease in the surface hydrophobicity is generally correlated with degree of elevation of the organic solvent tolerance level (Aono and Kobayashi 1997). The decrease in cell-surface hydrophobicity of the OST mutants is likely one of the mechanisms for tolerance to organic solvents by defending against penetration.

Among the organic solvents with log P_{ow} values of 2.7–5.5 (Table 4), those with the lower values were incorporated abundantly by *E. coli* cells. This phenomenon can explain why a more polar organic solvent is more toxic to microorganisms. This correlation is not the case in the phospholipid liposomes, as was described. This contrast suggests that biological membranes of viable cells are defended against organic solvents by some biological components or functions.

The cell surface of *E. coli* is an outer leaflet of the outer membrane. The outer leaflet is composed mainly of proteins and lipopolysaccharide. The OST mutants showed pleiotropic alterations in the envelope components. The most easily rationalized changes were found in the outer membrane components, as follows: loss of OmpF porin, and increased contents of peptidoglycan-bound lipoprotein and lipopolysaccharide. Lipopolysaccharide arrays only on the outer leaflet of the outer membrane. Loss of OmpF might indicate that organic solvents cross the outer membrane through the porin channels. The following explanation also seems plausible. Increase in the lipopolysaccharide content contributes to decreased hydrophobicity of the cell surface. Probably, the increase in lipoprotein content mechanically strengthens the outer membrane structure. It is likely that these changes are involved in improvement of organic solvent tolerance levels of OST mutants constructed from *E. coli*. Length and saturation of fatty acids were almost the

Table 2. Solvent tolerance levels of OST mutants derived from JA300

Solvents	log P_{ow}	Strain					
		JA300	OST3408	OST3410	OST3101	OST3301	OST3121
Diphenyl ether	4.2	+	+	+	+	+	+
n-Hexane	3.9	+	+	+	+	+	+
Propylbenzene	3.7	−	+	+	+	+	+
Cyclohexane	3.4	−	+	+	+	+	+
n-Pentane	3.3	−	−	−	+	+	+
p-Xylene	3.1	−	−	−	−	+/−	+
Toluene	2.6	−	−	−	−	−	−

+, growth; +/−, slight growth; −, no growth.
Susceptibility of each strain for the solvents was tested on LBGMg agar overlaid with a 2-mm-thick layer of each solvent.

Table 3. Cell-surface hydrophobicity of OST mutants of *E. coli*

Strain	Organic solvent tolerance level		MATH(%)[a]
	Index solvent	Index value	
JA300	*n*-Hexane	3.9	72.5
OST3408	Cyclohexane	3.4	50.1
OST3301	*n*-Pentane	3.3	26.9
OST3101	*P*-Xylene	3.1	12.9
OST3121	*P*-Xylene	3.1	10.0

[a] Suspensions of the microbial cells were well mixed with *n*-octane. Cells with hydrophobic cell surface are adherent to surfaces of *n*-octane droplets. The *n*-octane droplets coated with many cells are stable even after phase separation of the mixture. As a result, cells adhering to the droplets are removed from the water layer. MATH shows decrease in turbidity of the suspension by adhesion of cells to *n*-octane droplets. The value shown was a mean of determinations in dual experiments.

same among the parent and OST mutants, although it is reported that fatty acid composition of *P. putida* is altered by exposure to toluene (Weber et al. 1994).

Chemical analyses of surface layers of the OST mutants can be summarized as described. However, an additional explanation is possible because it was shown that OST3408 (Asako et al. 1997) and OST3410 (unpublished work), cyclohexane-tolerant mutants isolated independently from JA300, were defective in the *marR* gene as is described next.

Genes involved in determination of organic solvent-tolerant levels in *E. coli*

Successful construction of the OST mutants gave evidence that *E. coli* has genes involved in determining organic solvent tolerance levels. We analyzed *E. coli* genes involved in determination of the organic solvent tolerance level by the following three methods: conjugational analysis between two experimental strains with different tolerance levels; locus analysis of the cyclohexane tolerance determinant present in OST3408 and OST3410; and shotgun cloning of genes capable of elevating the organic solvent tolerance level of JA300.

Two strains of *E. coli*, JA300 [F^-, diphenyl ether-tolerant, *n*-hexane-sensitive (Hex^s)] and W2252 [HfrC, diphenyl ether-tolerant, *n*-hexane-tolerant (Hex^r)], were analyzed (Aono et al. 1994c,d). Hex^s was a hereditary characteristic that is mobile from the male to the female. The frequency of Hex^s was higher in the transconjugants than those of alleles of ordinary genetic markers, suggesting that several genes were probably involved cooperatively in determining the Hex^r phenotype found in JA300. These genes scored as *n*-hexane tolerant are tentatively called *ost* (organic solvent tolerance) genes. One *ost* gene is present between *leu* and *thr*. Hex^s and Hex^r can be transferred to each other by P1 transduction of this gene, *ostA*.

ostA is located at 1.1 min on the chromosome and neighbors to *pdxA* at a distance of 2–3 kb (Aono et al. 1994c). Nucleotide sequence of the 0–2.4 min region of the *E. coli* chromosome has been reported (Kohara et al. 1987; Yura et al. 1992). Gene *ostA* cloned by the gene walking method is likely identical to a 2352-bp open reading frame (ORF80) and *imp* gene (Sampson et al. 1989). A product of *ostA* was a 90-kDa outer membrane protein. Computer analysis suggests that OstA may enrich in β-sheet domains. A mutant *imp* gene made *E. coli* membrane permeable with maltodextrins. OstA might be a porin-like protein. In the *n*-hexane-sensitive strain, W2252, the promoter of the ostA gene seems to be inactivated by insertion of an IS (insertion) element (unpublished work).

Two genes, *ostB* and *ostC*, were cloned from chromosomal DNA of JA300 by their ability to improve the organic solvent tolerance level of W2252 (unpublished work). These genes improved the tolerance of *ostA*-defective derivative but not those of other strains, and form an operon mapped at 84.5 min on the chromosome of *E. coli*. *ostB* seems to encode a 26-kDa cytoplasmic protein having a helix-turn-helix motif like a GntR family protein (Gallegos et al. 1993). OstB probably regulates expression of *ostC*. *ostC* is thought to encode a 52-kDa inner membrane protein having 14 transmembrane domains. The putative OstC protein is homologous to AraE family proteins, indicating that this protein might be a H^+/drug antiporter. This gene might correspond to a gene already known as *bglT* (Prasad et al. 1973).

Three genes elevating the organic solvent tolerance of JA300 have been cloned by the shotgun method from the

Table 4. Incorporation of organic solvents by *E. coli* cells

Straina[a]	Amount of organic solvent binding to the cells[b] (μmol/mg of cellular protein)				
	n-Nonane (log P_{ow} 5.5)	Cyclooctane (4.5)	Cyclohexane (3.4)	*p*-Xylene (3.1)	Toluene (2.7)
JA300	<0.001	0.046	0.10	0.13	0.16
OST3408	<0.001	0.032	0.036	0.075	0.088
OST3121	<0.001	0.009	0.024	0.036	0.071

[a] Each organism was grown overnight in LBG medium at 37°C.
[b] The cell suspensions were overlaid with organic solvent and incubated for 1 h at 37°C. Organic solvents incorporated by the cells were extracted with chloroform and determined.

Table 5. Organic solvent tolerance levels of *tolC*-defective strains

Strain[a]	Plasmid	Growth in the presence of[b]						
		Decane (6.0)	Nonane (5.5)	Octane (4.9)	Diphenyl ether (4.2)	*n*-Hexane (3.9)	Cyclohexane (3.4)	*p*-Xylene (3.1)
JA300	pBluescript II	+	+	+	+	+	−	−
	pTolC	+	+	+	+	+	−	−
	pMarA	+	+	+	+	+	+	−
	pSoxS	+	+	+	+	+	+	−
	pRobA	+	+	+	+	+	+	−
JA300T	pBluescript II	+	−	−	−	−	−	−
	pTolC	+	+	+	+	+	−	−
	pMarA	+	−	−	−	−	−	−
	pSoxS	+	−	−	−	−	−	−
	pRobA	+	−	−	−	−	−	−
OST3408T	pBluescript II	+	−	−	−	−	−	−
	pTolC	+	+	+	+	+	+	−
	pMarA	+	−	−	−	−	−	−
	pSoxS	+	−	−	−	−	−	−
	pRobA	+	−	−	−	−	−	−
Bacillus subtilis	GSY1026[c]	+	−	−	−	−	−	−

[a] JA300T and OST3408T are *tolC*-defective derivatives of JA300 and OST3408, respectively.
[b] +, growth; −, no growth; log P_{ow} value of the organic solvent is shown in parentheses.
[c] *B. subtilis* GSY1026 was used as one typical example of gram-positive bacteria.

JA300 chromosome itself. These genes, *marA*, *robA*, and *soxS*, were effective to elevate the tolerance level of several strains only when transformed using high copy vectors (Nakajima et al. 1995a,b; Asako et al. 1997). Products of *marA* (locus 34.1 min), *robA* (99.8 min), and *soxS* (92.2 min) are known to be cytoplasmic proteins positively regulating expression of several genes belonging to the *mar-sox* regulon (Cohen et al. 1993; Gruer and Guest 1994; Ariza et al. 1995). *marA* and *soxS* confer tolerance on *E. coli* to multiple antibiotics and superoxide anion. Expression of genes conferring organic solvent tolerance of *E. coli* might be positively regulated by these regulatory proteins (Aono et al. 1995). When *marA*, *robA*, or *soxS* copy was multiplied by using a high copy vector, the transformed cells of JA300 enhanced expression of *mar-sox* regulon genes and acquired cyclohexane tolerance. The transformed cells produced high amounts of AcrA and TolC (Aono et al. 1998) consisting of an efflux pump system for hydrophobic compounds (Ma et al. 1995; Fralick 1996). Also, JA300 cells exposed to methylviologen generating O_2^- in the culture acquired cyclohexane tolerance. The anion is known to enhance expression of *soxS* (Wu and Weiss 1992).

Cyclohexane-tolerant mutants, OST3408 and OST3410, are defective in *marR*, a repressor protein for *mar* operon including *marA* (Asako et al. 1997). In these mutants, levels of AcrA and TolC are increased (Aono et al. 1998) like JA300 cells transformed with *marA*, *soxS*, or *robA*. AcrA, AcrB, and TolC are constituents of an efflux pump driven by the proton motive force (Ma et al. 1995; Fralick 1996). This efflux pump system is a major pump exporting various hydrophobic compounds in *E. coli* (Okusu et al. 1996). Genes *acrA* and *acrB* form one transcriptional unit. AcrB is likely to be produced at a high level although we did not detect AcrB by sodium dodecyl sulfate-(SDS-) polyacrylamide gel electrophoresis, probably because of its high molecular mass and complex folding in the membrane. It is likely that this AcrAB efflux pump occupies the most important position to determine the microbial organic solvent tolerance level. It is reported that the *n*-hexane-tolerant *E. coli* strain lost the tolerance by deletion of the *acrAB* loci (White et al. 1997). We also found that organic solvent tolerances of *acrA*-defective strains were extremely low. In addition, the tolerance level of a *tolC*-defective mutant was extremely low (Table 5), and was restored by transforma-

tion of the wild *tolC* gene (Aono et al. 1998). The tolerance levels of the *acrA*- or *tolC*-defective mutant were never improved by transformation with *marA*, *robA*, or *soxS*, indicating that these genes elevated the tolerance level via function of the AcrAB-TolC efflux pump.

Active efflux of toluene is reported to occur in a toluene-tolerant *P. putida* strain (Isken and de Bont 1996). This observation shows that an energy-dependent export system might be responsible for the toluene tolerance. An efflux pump driven by the proton-motive force, the MexA-MexB-OprM system, is known to exist in *P. aeruginosa* (Poole et al. 1993; Li et al. 1995). This pump, or a similar system, might discharge organic solvent molecules incorporated into the cells and play an important role in maintenance of the organic solvent tolerance of *P. aeruginosa* and *P. putida*. In fact, we found that the organic solvent tolerance level of *P. putida* PpY101 was slightly improved by transformation with the *mexA-mexB-oprM* operon cloned from *P. aeruginosa* PAO3292 (unpublished work). Efflux pump systems driven by the proton motive force seem to contribute to determining the intrinsic levels of organic solvent tolerance in a wide range of gram-negative bacteria.

It is unclear how some cloned genes described here (*ostA, ostB, ostC*) improve the organic solvent tolerance levels of the host cells. Microorganisms might have various strategies to improve the tolerance levels. Further studies on mechanisms to improve organic solvent tolerance levels are under way.

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