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Organic Solvent Tolerance of *Escherichia coli* Is Independent of OmpF Levels in the Membrane

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The organic solvent tolerance of *Escherichia coli* was measured under conditions in which OmpF levels were controlled by various means as follows: alteration of NaCl concentration in the medium, transformation with a stress-responsive gene (*marA*, *robA*, or *soxS*), or disruption of the *ompF* gene. It was shown that solvent tolerance of *E. coli* did not depend upon OmpF levels in the membrane.

We previously constructed *Escherichia coli* mutants displaying improved organic solvent tolerance (3). *E. coli* JA300 (19), used as the parent, produced OmpF porin protein in the membrane even when grown in the presence of 1% NaCl. In contrast, the levels of OmpF protein were markedly decreased in the mutants because of mutations in *marR* (4, 9). It is reported that OprF porin protein is absent in a toluene-tolerant mutant of *Pseudomonas aeruginosa* (22). Hydrophobic β-lactam antibiotics pass through OmpF channels faster than through OmpC channels (28). It seemed likely that organic solvent molecules also could pass through the OmpF porin. Therefore, it was supposed that the decreased levels of OmpF or loss of OmpF might contribute to improvement of organic solvent tolerance in *E. coli*, as suggested for acquisition of multiple antibiotic resistance (2, 6). In this study, this possibility was explored by measuring the organic solvent tolerance of *E. coli* under conditions in which OmpF synthesis was controlled by various means.

First, we controlled OmpF synthesis in JA300 by growth under different salinity conditions. OmpF synthesis is repressed under conditions of high environmental osmolarity (13). This osmoregulation is mediated mainly by an increase in expression of *micF* RNA, an antisense RNA that inhibits OmpF translation. The extent of osmoregulation was evaluated for JA300 grown in the medium that we have usually used. A membrane fraction was prepared from sonicated lysate of the cells grown in modified Luria broth (LBGMg) (1% [wt/vol] Bacto Tryptone [Difco Laboratories, Detroit, Mich.], 0.5% Bacto Yeast Extract [Difco], 0.1% glucose, and 10 mM $MgSO_4$) containing or not containing 1% (wt/vol) NaCl. JA300 produced a considerable amount of OmpF in the presence of NaCl, although the amount was less than that produced in the absence of NaCl (Fig. 1 and Table 1). The effect of NaCl on the OmpF level of JA300 carrying a vector plasmid, pBluescript II SK(+) (Toyobo Biochemical Inc., Osaka, Japan; hereafter pBS) was similar to that found in the host cell (results not shown).

LBGMg agar on which JA300 was plated was overlaid with a ca. 2-mm layer of an appropriate organic solvent and incubated at 37°C overnight. When JA300 grew confluently, it was considered that JA300 was tolerant of the organic solvent overlaying the agar (5). The toxicity of the organic solvent is reflected by its log P_{OW} value (5, 15), shown in Table 1. This value is inversely correlated with the toxicity of organic solvent. Here, log P_{OW} is the common logarithm of P_{OW}, the partition coefficient of the organic solvent between *n*-octanol and water. Growth without NaCl did not greatly lower the organic solvent tolerance level of JA300, although the OmpF level was high, as described above. We found that growth in the absence of NaCl reduced the organic solvent tolerance of JA300(pBS), compared with that grown in the presence of 1% (wt/vol) NaCl. This difference was observed probably because less growth occurred on the agar not containing NaCl.

Second, the OmpF levels were reduced by overexpression of *marA*, *robA*, or *soxS*. Transcriptional activators, MarA, Rob, and SoxS, positively regulate expression of *mar-sox* regulon genes including *micF* (8, 10, 17). Overproduction of these proteins triggered low production of OmpF. We intended to control OmpF synthesis through the repression brought about by overexpression of the stress-responsive genes and by growth at high osmolarity (salinity). The plasmids used here, pMarA, pRob, and pSoxS, were referred to previously as pHA105 (9), pOST42BR (26), and pHc3R (27), respectively.

The level of OmpF was reduced upon introduction of one of the plasmids (Fig. 1 and Table 1). The extent of repression differed depending on the plasmids carried and NaCl concentration. The repression caused by pMarA was independent of NaCl concentration. That caused by pRob was high in the

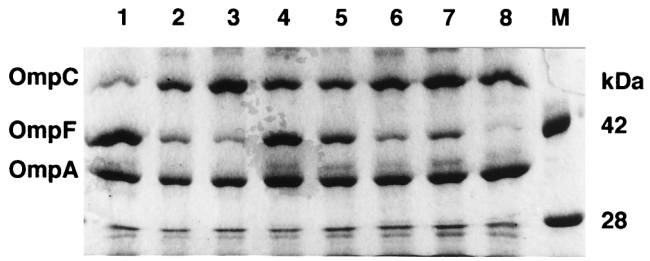

FIG. 1. Sodium dodecyl sulfate-urea-polyacrylamide gel electrophoresis of envelope protein. JA300 cells transformed with the plasmids were grown in LBGMg medium. The cells were broken by sonication. Membrane fractions were extracted with 0.5% sodium *N*-lauroylsarcosine at room temperature (11). Insoluble proteins (45 μg) were electrophoresed on 0.1% sodium dodecyl sulfate–4 M urea–10% (wt/vol) polyacrylamide gels (1). Protein was stained with Coomassie Brilliant Blue R-250. Lanes: M, molecular size markers; 1 to 4, growth in the absence of NaCl; 5 to 8, growth in the presence of 1% (wt/vol) NaCl; 1 and 5, no plasmid; 2 and 6, pMarA; 3 and 7, pRob; 4 and 8, pSoxS. Bands containing OmpC, OmpF, and OmpA are shown.

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TABLE 1. Organic solvent tolerance levels of *E. coli* JA300 and its derivative

Strain	Plasmid	1% (wt/vol) NaCl[a]	Growth on LBGMg agar overlaid with[b]:						OmpF level[c] (%)
			DE 4.2	*n*Hex 3.9	*n*Hex/CH 3.7	CH 3.4	CH/*p*X 3.3	*p*X 3.1	
JA300	None	−	+	+	−	−	−	−	100
		+	+	+	−	−	−	−	38
	pBS	−	+	−	−	−	−	−	NT
		+	+	+	−	−	−	−	NT
	pMarA	−	+	+	+	+	−	−	13
		+	+	+	+	+	−	−	11
	pRob	−	+	+	+	+	−	−	7
		+	+	+	+	+	−	−	19
	pSoxS	−	+	+	+	+	−	−	73
		+	+	+	+	+	−	−	<1
JOF501	None	+	+	+	−	−	−	−	0

[a] The organisms were grown on LBGMg agar containing or not containing NaCl (1% [wt/vol]).

[b] Abbreviations: DE, diphenyl ether; *n*Hex, *n*-hexane; CH, cyclohexane; *p*X, *p*-xylene; *n*Hex/CH and CH/*p*X, solvent mixtures consisting of *n*-hexane–cyclohexane and cyclohexane–*p*-xylene (1:1, vol/vol), respectively. The number shown under the solvent name represents the log P_{OW} value of the solvent. The value was calculated by the addition rule (21) with log P_{OW} calculation software, ClogP, version 1.0.3 (Bio Byte Corporation, Claremont, Calif.). The approximate values were estimated for solvent mixtures on the basis of the calculation rule (20).

[c] The level of OmpF was estimated by image analysis with Bio Image Intelligent Quantifier version 2.1.2a software (Bio Image Systems Corporation, Ann Arbor, Mich.); NT, not tested.

absence of NaCl. In contrast, pSoxS severely repressed OmpF production in the presence of NaCl and slightly repressed it in the absence of NaCl. Consequently, various levels of OmpF production were achieved in JA300 cells.

We reported that overexpression of the genes improved the organic solvent tolerance of *E. coli*, based on the tolerance measured by monitoring growth in the presence of NaCl (9, 26, 27). The overexpression made JA300 grown without NaCl as highly tolerant as that grown with NaCl (Table 1). As far as examined, the organic solvent tolerance of each transformant did not differ between cells grown with NaCl and those grown without NaCl. It is particularly notable that JA300(pSoxS) grown without NaCl produced a high level of OmpF and was tolerant of cyclohexane, indicating that the increased level of OmpF did not reduce tolerance of cyclohexane. These results suggest that organic solvent tolerance levels are not directly related to OmpF levels.

The OmpF levels were controlled via *micF* expression in the experiments described above. These controls are indirect for OmpF production. In particular, growth of the organisms under different conditions such as salinity might cause unexpected effects on the cell membrane structures other than OmpF production. Finally, we constructed an *ompF* disruptant from JA300 and examined the solvent tolerance level. From *E. coli* RK4786 (14), *ompF*::Tn*5* was transduced into JA300 by generalized transduction with P1*kc* (25). An OmpF-nonproducing transductant (JOF501) was selected from among kanamycin-resistant clones grown on LBGMg agar containing kanamycin (50 μg/ml). This P1 transductant did not display OmpF in the membrane at all, regardless of NaCl concentration (results not shown). The organic solvent tolerance level of the *ompF* disruptant, measured on LBGMg agar containing NaCl, was identical to that of JA300 (Table 1). The *ompF* disruption did not result in improvement of the organic solvent tolerance.

The organic solvent tolerance of JA300 was not altered to any detectable extent as a direct result of a decreased or increased level of OmpF in the membrane or its absence, except for JA300(pBS). The extremely low level of organic solvent tolerance of the *tolC* disruptant derived from JA300 is not improved by transformation with pMarA, pRob, or pSoxS (7), although the OmpF levels decreased in the transformants (results not shown). This fact supports the conclusion described above. It is likely that the solvent tolerance of the mutants or transformants is improved mainly by elevated expression of *acrAB* and *tolC* (7), not by repression of OmpF synthesis. Genes *acrAB* and *tolC* encode AcrA, AcrB, and TolC, consisting of a proton motive force-dependent efflux pump to extrude multiple antibiotics (12, 24). Recently, energy-dependent efflux systems extruding organic solvents have been reported to contribute to the organic solvent tolerances of *E. coli* (7, 32), *P. aeruginosa* (23, 29), and *Pseudomonas putida* (16, 18, 30, 31). Probably the AcrAB and TolC efflux system plays the most important role in organic solvent tolerance in *E. coli*.

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