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Novel Toluene Elimination System in a Toluene-Tolerant Microorganism

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In studies of *Pseudomonas putida* IH-2000, a toluene-tolerant microorganism, membrane vesicles (MVs) were found to be released from the outer membrane when toluene was added to the culture. These MVs were found to be composed of phospholipids, lipopolysaccharides (LPS), and very low amounts of outer membrane proteins. The MVs also contained a higher concentration of toluene molecules (0.172 ± 0.012 mol/mol of lipid) than that found in the cell membrane. In contrast to the wild-type strain, the toluene-sensitive mutant strain 32, which differs from the parent strain in LPS and outer membrane proteins, did not release MVs from the outer membrane. The toluene molecules adhering to the outer membrane are eliminated by the shedding of MVs, and this system appears to serve as an important part of the toluene tolerance system of IH-2000.

Organic solvents are very toxic to microorganisms. Studies have shown that toluene destroys the inner membrane of gram-negative bacteria (7, 16, 35). However, Inoue and Horikoshi discovered the toluene-tolerant strain *Pseudomonas putida* IH-2000 (11). It has been demonstrated that the degree of toxicity of an organic solvent corresponds to its log P_{ow} value, which is the logarithm of the partition coefficient of the organic solvent between *n*-octanol and water (11). Organic solvents with a lower log P_{ow} have higher toxicity to microorganisms (12). Many reports have described *Pseudomonas* strains that were toluene tolerant (6, 14, 27, 32). Organic solvent-tolerant microorganisms have attracted interest due to the possibility of applying them to persolvent fermentation of water-insoluble compounds (5).

We have studied the mechanisms of toluene tolerance in *P. putida* IH-2000, and there have been various reports about such mechanisms in *Pseudomonas* species. The mechanisms of organic solvent tolerance have been shown to involve active efflux pumps (13, 20, 26, 30); low cell hydrophobicity, which serves to keep solvent molecules away from the cell surface (2, 21); and low fluidity of the bacterial membrane, to protect the cell from the solvent (8, 10, 17). In these studies, many mutants either tolerant or sensitive to organic solvents have been isolated from *Pseudomonas* or *Escherichia coli* (4, 15, 29). These mutants showed phenotypic changes not only in solvent tolerance levels but also in antibiotic resistance levels (4, 15). We have reported the isolation of a toluene-sensitive mutant, strain 32, which showed unchanged antibiotic resistance levels, obtained through transposon mutagenesis using Tn*5*. The gene disrupted by insertion of Tn*5* was identified as *cyoC*, which is one of the subunits of cytochrome *o* (21). The outer membrane protein profile and lipid composition of lipopolysaccharides (LPS) of strain 32 were found to differ from those of IH-2000. Furthermore, the cell surface hydrophobicity of strain 32 was greater than that of IH-2000 (21). Even if the cell surface shows very low hydrophobicity, organic solvent molecules intercalate into and accumulate in the cell membrane and finally disrupt its structural integrity (34). The toluene molecules adhering to the cell membrane must be eliminated. Active efflux pump systems have been reported elsewhere to serve as one of the mechanisms of toluene elimination (13, 20, 26, 30).

We report here a novel toluene tolerance system in *P. putida* IH-2000 which involves the elimination of toluene from the outer membrane through the release of membrane vesicles (MVs) composed of phospholipids and LPS. This defense system appears to represent a novel function of the outer membrane of gram-negative bacteria.

MATERIALS AND METHODS

Culture medium and strains. The toluene-tolerant microorganism *P. putida* IH-2000 was isolated by Inoue and Horikoshi (11). The toluene-sensitive mutant strain 32 derived from strain IH-2000 was isolated in a previous study (21). *P. putida* type strain IAM1236^T, which is sensitive to toluene, was employed as a standard strain. *Pseudomonas* strains were aerobically grown at 30°C in modified Luria-Bertani (LB) medium (21) (LB-Mg medium) consisting of 1.0% (wt/vol) tryptone peptone (Difco Laboratories, Detroit, Mich.), 0.5% (wt/vol) yeast extract (Difco), 1.0% (wt/vol) NaCl, and 10 mM $MgSO_4 \cdot 7H_2O$ per liter. Toluene was added to the medium at a final concentration of 10% (vol/vol). For cultivation of bacteria on plates, the medium was supplemented with 1.5% (wt/vol) agar. Toluene was purchased from Wako Pure Chemical Industries (Osaka, Japan).

Electron microscopy. Electron microscopy was carried out according to the method described in reference 1. Each culture was filtered, and the cells were collected on an HA membrane (Millipore Co., Ltd.). The membrane was cut and mounted on a Teflon support placed on the end of a plunger. The plunger was dropped from a height of 10 cm onto a gold-coated pure copper block precooled in liquid nitrogen. The membrane frozen by metal block contact was dropped into precooled acetone (−80°C) containing 2% (wt/vol) OsO_4 and kept at the same temperature (−80°C) for 36 h in this solution. Thereafter, the solution was warmed stepwise to room temperature (−20°C, 2 h, 4°C, 2 h; room temperature, 2 h), the membrane was washed three times, and then it was infiltrated with 100% acetone. The treated membrane was embedded in epoxy resin (Epon 812). Thin sections were cut with a diamond knife on a Reichert Ultracut S (Leica Co.) ultramicrotome. After being stained with uranyl acetate and lead citrate, they were examined with a JEM-1210 electron microscope at 80 kV.

Fatty acid analysis and toluene measurement. The samples of freeze-dried MVs or bacterial cells were heated in 5% (wt/vol) methanolic HCl at 100°C for 3 h. The resulting fatty acid methyl esters (FAMEs) were extracted twice with *n*-hexane and concentrated under a stream of nitrogen gas. The FAMEs were analyzed using a gas-liquid chromatograph (model GC-380; GL-Science). The toluene molecules adhering to the MVs or to the bacterial cells were collected by extracting the samples twice with chloroform. The amount of toluene in the extracts was measured using a gas-liquid chromatograph (model GC-380).

Preparation of MVs. MVs were prepared as described in reference 19. Cells of strain IH-2000 were grown aerobically in 2 liters of LB-Mg medium containing 10% (vol/vol) toluene for 6 h at 30°C. After the cells were removed from the culture medium by centrifugation, toluene in the supernatant was removed by

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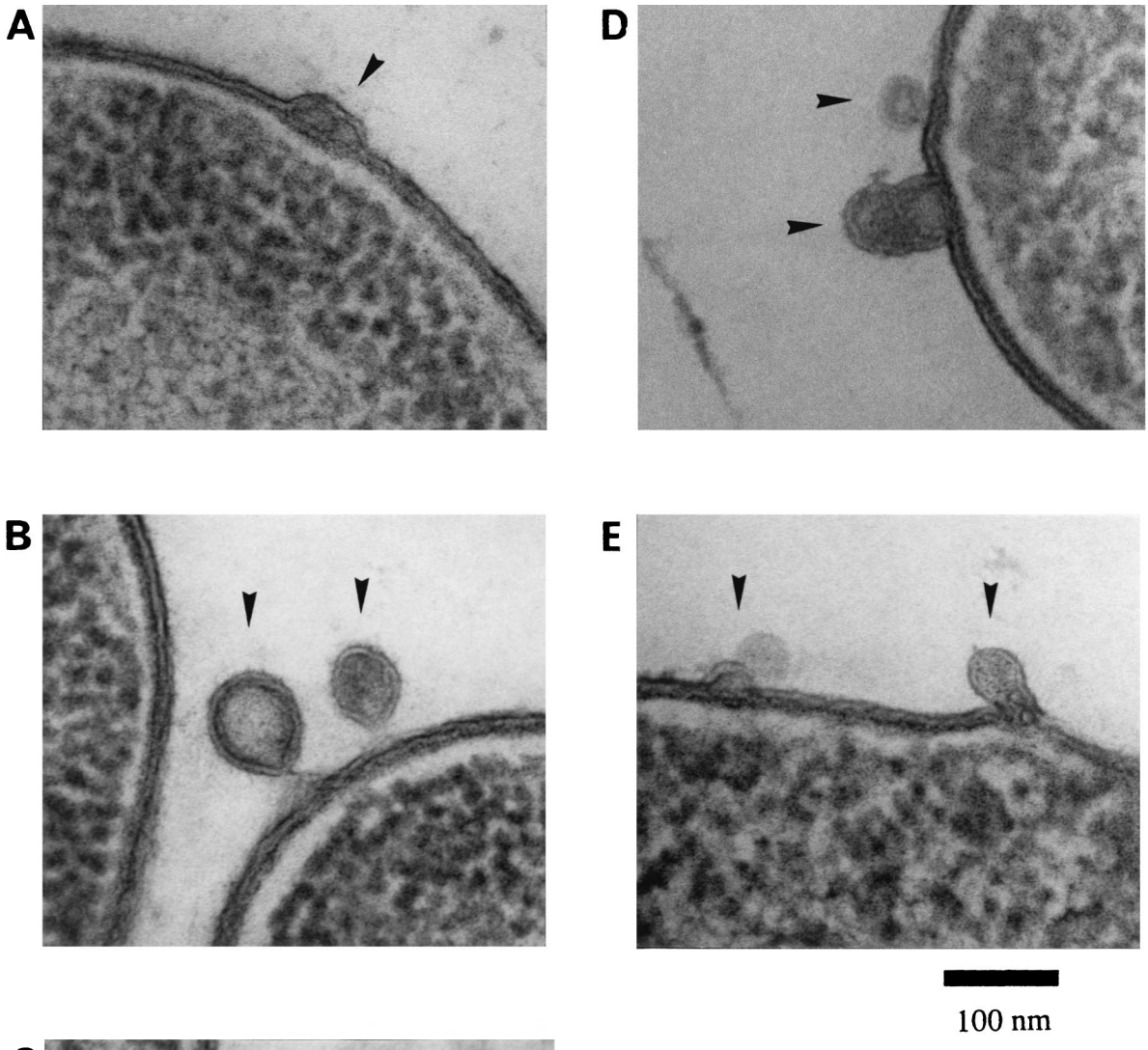

FIG. 1. The formation of MVs (indicated by arrowheads) from the outer membrane of IH-2000 was observed when cells were grown in a medium containing toluene. MV formation would start with the swelling of the outer membrane (A). The outer membrane was not damaged by MV formation (B). The MVs released were also detected, and there were no electron-dense materials enclosed (C). Incomplete MVs (indicated by arrowheads) were produced by a toluene-sensitive mutant, strain 32 (D and E). The MVs of strain 32 were smaller than those of IH-2000 and differed in morphology. Bar = 100 nm.

evaporation at 35°C. Then, the supernatant was filtered using a 0.22-μm-pore-size filter (Whatman, Inc., Clifton, N.J.) twice. The MVs were harvested by centrifugation (100,000 × *g*, 3 h, 4°C), washed twice with cold 0.8% NaCl, and suspended in 500 μl of 0.8% (wt/vol) NaCl. Electron microscopy showed that this MV fraction contained the flagella of IH-2000 (data not shown).

Hydrophobicity assay of MVs. The hydrophobicity assay was carried out using the modified method of bacterial adhesion to hydrocarbon (21). Sixty microliters of *p*-xylene was added to 400 μl of MV fraction. The bilayer solution was vortexed for 1 min and left for 10 min at room temperature. The phospholipids were extracted from the MV fraction before and after vortexing by the method of Bligh and Dyer as described in reference 32. The phosphorus content of each phospholipid fraction was determined by the method given in reference 21, and hydrophobicity was calculated from the following equation: hydrophobicity (%) = (1 − Aa/Ab) · 100 (Aa, phosphorus content after the vortexing; Ab, phosphorus content before the vortexing).

RESULTS

Production of MVs from the outer membrane of IH-2000. We have been studying the toluene tolerance system of *P. putida* IH-2000 and have isolated a toluene-sensitive mutant of this strain, strain No.32 (21). In the present study, using transmission electron microscopy (TEM), we examined the cell surface of IH-2000 and strain 32 cells grown aerobically at 30°C in LB-Mg medium containing toluene (Fig. 1). MVs were found to be produced from the outer membrane of IH-2000 cells

TABLE 1. Fatty acid composition[a] of MVs

Fatty acid[b]	Origin[c]	Amt of lipids	
		nmol/ml of culture	Ratio (%)
C10:0-3OH	LPS	0.100	6.1
C12:0	LPS	0.161	3.8
C12:0-2OH	LPS	0.437	16.6
C12:0-3OH	LPS	0.585	6.2
C16:0	PL	0.468	22.2
C16:1t	PL	0.365	17.8
C16:1	PL	0.163	13.8
C18:0	PL	0.033	1.2
C18:1t	PL	0.116	4.4
C18:1	PL	0.210	7.9
Total		2.64	100

[a] The samples of freeze-dried MVs or bacterial cells were heated in 5% (wt/vol) methanolic HCl at 100°C for 3 h. The resulting FAMEs were extracted twice with *n*-hexane and concentrated under a stream of nitrogen gas. The FAMEs were analyzed using a gas-liquid chromatograph (model GC-380; GL-Science).

[b] C10:0-3OH, 3-hydroxyldecanoic acid; C12:0, dodecanoic acid; C12:0-2OH, 2-hydroxyldodecanoic acid; C12:0-3OH, 3-hydroxyldodecanoic acid; C16:0, hexadecanoic acid; C16:1t, *trans*-hexadecanoic acid; C16:1, *cis*-hexadecanoic acid; C18:0, octadecanoic acid; C18:1t, *trans*-octadecanoic acid; C18:1, *cis*-octadecanoic acid.

[c] These fatty acid molecules were found in the membrane of IH-2000 (17). PL, phospholipid.

grown in the toluene-containing medium (Fig. 1A to C). The diameter of the MVs was in the range of about 80 to 100 nm. The MVs were not detected when IH-2000 cells were grown in the same medium without toluene (data not shown). Although MVs were also observed on the cell surface in the case of strain 32 after incubation with toluene for 1 h, these MVs were incomplete (Fig. 1D and E), and no free MVs were observed by TEM. The diameter of these MVs was about 40 nm, substantially less than the diameter of those of IH-2000. In the case of the *P. putida* type strain, IAM1236^T, which is sensitive to toluene, such MV formation was not observed in either the presence or the absence of toluene (data not shown).

Analysis of MVs. To examine the relationship between MV formation and the toluene tolerance of IH-2000, the properties of the MVs were further investigated. In the preparation of MV fractions from cultures of strains IH-2000 and strain 32, we were able to obtain MVs only in the case of IH-2000. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the MV fraction of IH-2000 showed bands of LPS, a major 32-kDa protein, and a few minor proteins (data not shown). The N-terminal amino acid sequence of the 32-kDa protein was found to be ALTVNTNTTSLGVQKNLNRASEALSTS MTRLSS, showing 96% identity with the flagellin of *P. putida* (33). It seems likely that this flagellin has no relationship with the MVs, since many flagella were detected in the MV fraction by electron microscopy (data not shown). Fatty acid analysis revealed that the MVs contained lipids characteristic of the membrane phospholipids and LPS of IH-2000 (Table 1). Toluene molecules were also present in the MVs at a concentration of 0.172 ± 0.012 mol/mol of lipid (± standard deviation [SD]; $n = 3$). The MVs consisted of phospholipids, LPS, a small amount of protein, and toluene. The hydrophobicity of MVs was 39.0 ± 1.2 (± SD; $n = 3$).

Toluene molecules adhering to the cell surface. Do toluene molecules accumulate in the MVs produced by strain IH-2000? The amount of toluene molecules adhering to the cells was measured to address this question. Strains IH-2000 and 32 were each grown in 1 liter of LB-Mg medium at 30°C until the optical density at 660 nm reached 0.4, and then toluene was added at a final concentration of 10% (vol/vol). Cells were harvested from 100 ml of the culture at 0.5, 1.0, and 2.0 h after the addition of toluene. The cells were washed with 100 ml of cold 0.8% (wt/vol) NaCl twice and then suspended in 1 ml of cold 0.8% (wt/vol) NaCl. Figure 2 shows the changes in the amount of toluene molecules adhering to the cells or MVs and the amount of the fatty acid of MVs in the culture. We calculated the amount of toluene per lipid in this study. The phospholipid was one of the major components of MVs, and toluene molecules would be intercalated into outer and inner membranes of IH-2000 because the growth rate of IH-2000 in the medium containing toluene was not as high as that of IH-2000 in the medium not containing toluene. In the case of strain IH-2000, the amount of toluene adhering to the cells was largest, 0.077 ± 0.0066 mol/mol of lipid (± SD; $n = 3$), 0.5 h after the addition of toluene. In the period 1 to 3 h after the addition of toluene, the amount adhering to the cells remained constant at about 0.026 mol/mol of lipid (Fig. 2). On the other hand, the amount of toluene adhering to the strain 32 cells continued to increase from 0.188 mol/mol of lipid, about twice as much as that in the case of IH-2000, to 1.03 mol/mol of lipid in the period from 0.5 to 2.0 h after the addition of toluene.

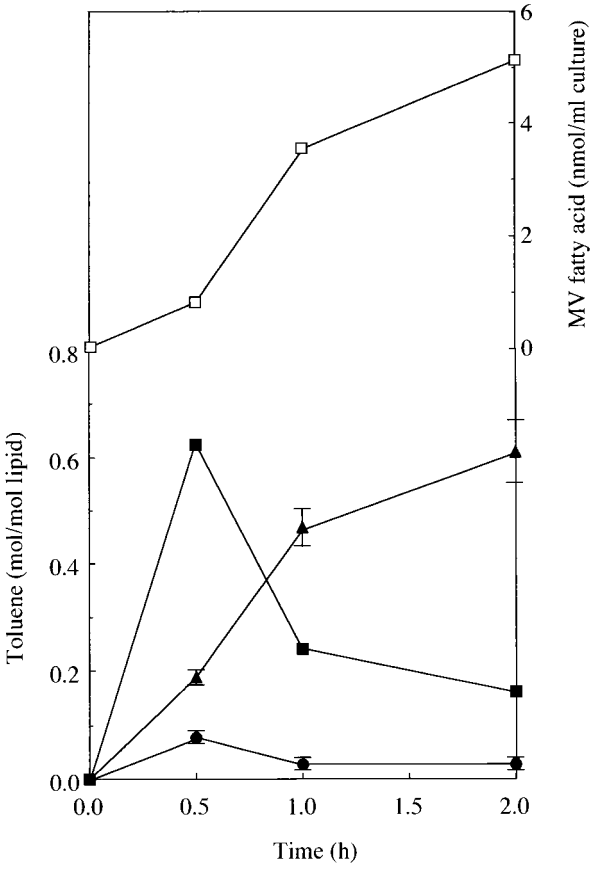

FIG. 2. Toluene molecules adhering to IH-2000 (●) or strain 32 (▲) cells or MVs (■) and the amount of fatty acids in MVs (□) in the culture. Toluene was added to a culture of strain IH-2000 or 32 (time = 0). Then the amount of toluene molecules adhering to the cells was measured, and the amount per fatty acid chain was calculated because the MVs were found to be composed of phospholipids and LPS. Error bars show means ± SDs ($n = 3$). The MV fractions were periodically prepared as described in Materials and Methods from the culture of IH-2000.

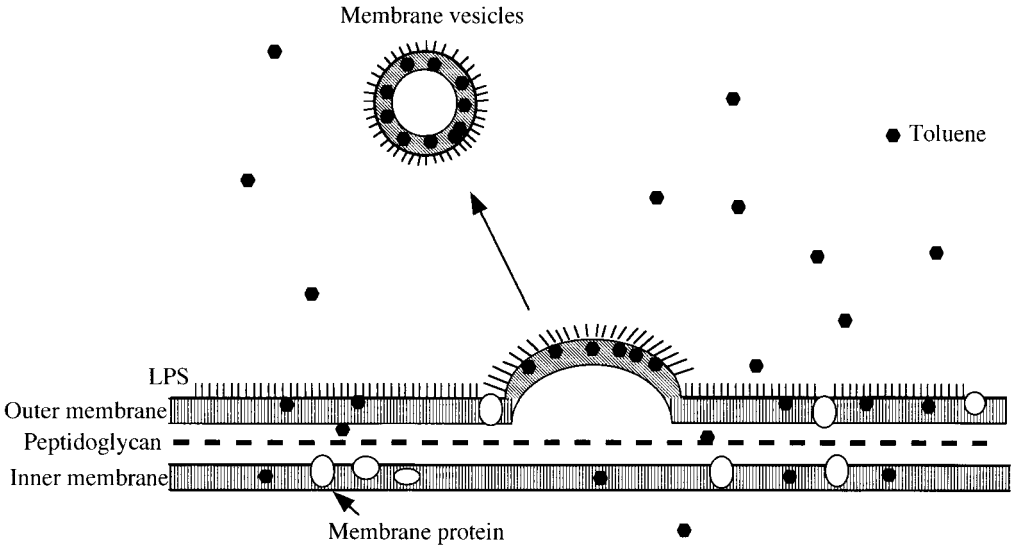

FIG. 3. Model of toluene elimination by production of MVs in strain IH-2000. Toluene molecules rapidly adhere to the outer membrane. The toluene molecules are stable in the hydrophobic region of the membrane. The MVs are formed from the outer membrane, and these serve to eliminate toluene molecules from the cell. The MVs released are not likely to fuse to other cells because the surface of the MVs is covered with the polysaccharide chains of LPS.

The MVs were detected at 0.5 h after the addition of toluene. The amount of fatty acids of MVs in the culture increased and showed 5.2 nmol/ml of culture at 2.0 h after the addition of toluene. The amount of toluene associated with the MVs decreased from 0.625 mol/mol of lipid to 0.16 mol/mol of lipid in the period from 0.5 to 2.0 h after the addition of toluene.

DISCUSSION

In this study, we observed MV formation on the outer membrane of cells of both strain IH-2000 and the toluene-sensitive mutant strain 32, when toluene was added to the culture. Although there have been several reports on TEM observations of the cell surface of bacteria grown in a medium containing an organic solvent or treated with an organic solvent (3, 6, 7, 16, 29, 35), such MV formation has not been reported. Also, *P. putida* IAM1236^T did not produce MVs when toluene was added to the culture in the present study. Therefore, it is evident that MV formation is not the result of destruction of the outer membrane by toluene molecules. It appears to be a characteristic response of strain IH-2000 to toluene molecules. The MVs were found to consist of phospholipid, LPS, a small amount of protein, and toluene (Table 1). The amount of toluene associated with the MVs was greater than the amount adhering to the cells, and the number of adherent toluene molecules decreased with the passage of time (Fig. 2). The MVs were detected only within the first hour after the addition of toluene. The decrease in the amount of toluene molecules adsorbed to the cells is likely due to their elimination through the production of MVs (Fig. 2). The number of toluene molecules adsorbed to the cells of toluene-sensitive mutant strain 32 0.5 h after the addition of toluene was about twice as much as that in the case of IH-2000 (Fig. 2). The adsorption of toluene molecules to the cell surface depended on cell surface hydrophobicity in the first 0.5 h. When the cell surface hydrophobicity of each of these strains was examined by a method involving measurement of bacterial adhesion to hydrocarbon and by a hydrophobic interaction chromatography method, the index value obtained for strain 32 by each method was indicative of a degree of hydrophobicity twice that of IH-2000 (21). Strain 32 failed to produce MVs, which serve to eliminate toluene molecules from the cell surface, and the amount of toluene molecules adsorbed to the cell surface increased with time (Fig. 2).

The amount of toluene associated with the MVs showed a maximum, 0.625 mol/mol of lipid, at 0.5 h after the addition of toluene (Fig. 2) and then decreased to 0.166 mol/mol of lipid at 2.0 h after the addition of toluene, which was almost the same as the 0.172 mol/mol of lipid at 6.0 h after the addition of toluene. This decrease showed that the amount of toluene molecules associated with MVs depended on the amount of that of IH-2000. The hydrophobicity of MVs was (39.0 $\pm$ 1.2)%, which was 1.2 times as great as that of IH-2000, 32.7% (21). The increase of hydrophobicity was so minor that there would not be a difference between the rates of permeation of toluene molecules into MVs and that into the cell membrane of IH-2000. Therefore, the difference in the amount of toluene molecules between MVs and IH-2000 would not be caused by the excess intercalation to MVs after release from the cell membrane. It was reported elsewhere that the short alkane formed an alkane region in the geometric center of the bilayer of the phospholipid (24) and altered the hydrophobic interior (25). The MVs may contain the regions changed by high levels of toluene and containing more toluene molecules than other regions. We conclude that, in strain IH-2000, toluene molecules adsorbed to the cells accumulate in MVs and are thereby eliminated.

The amount of fatty acids of MVs released into the culture from IH-2000 was 5.2 nmol/ml of culture at 2 h after the addition of toluene (Fig. 2). When the MV fraction was prepared from the culture with toluene for 6 h, the fatty acid level of MVs was 2.64 nmol/ml of culture. The MVs were detected in the emulsion phase of the culture (data not shown), and this would cause the decrease of MVs in the culture with toluene for 6 h. The total amount of MVs released into the whole culture was still unknown.

It has been reported elsewhere that bacterial outer MVs are produced by some pathogens as a means of releasing toxins (9, 18, 19, 22, 28, 36). The pathogens constantly produce MVs consisting of the toxins, phospholipids, LPS, outer membrane

proteins, and DNA (18, 19, 22). In these studies, it was concluded that the MVs function as an enzyme or toxin secretion system. These reports also demonstrate that MV formation is not so burdensome for the bacteria. The mechanisms of MV production, however, are still unknown. Strain 32 formed incomplete MVs (Fig. 1D and E) and could not produce free MVs. We have reported that the fatty acid composition of the LPS in strain 32 differs from that in the parent strain IH-2000 (21). LPS was found to be a component of the MVs, and changes in the lipid composition of LPS are likely to affect the formation of the MVs. Further study is needed to understand the formation of these MVs.

Figure 3 shows a model of the toluene elimination system of IH-2000. In the case of this strain, the cell surface shows very low hydrophobicity; however, this cannot stop the toluene molecules from breaking through into the cytoplasmic membrane. The toluene molecules in the outer membrane of IH-2000 appear to be eliminated through the production of MVs, which serve to protect the inner membrane, which contains many important proteins such as those involved in respiration. This model seems to be almost ideal for tolerance to organic solvents. Those organic solvents which are immiscible with water are very unstable in the water phase and adhere to hydrophobic material such as the lipid layers in cells. If the free toluene molecules were eliminated from the cells, these toluene molecules would immediately adhere to the cells again. The toluene molecules in this model are strongly intercalated in the lipid layer of the MVs and would not return to the cells. The outer membrane of gram-negative bacteria has been regarded as only a barrier (23). The results presented in this paper show that the outer membrane of gram-negative bacteria is sometimes not a simple barrier but an active eliminator of a toxic organic solvent in extremophiles.

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