Membrane Vesicle Formation as a Multiple-Stress Response Mechanism Enhances *Pseudomonas putida* DOT-T1E Cell Surface Hydrophobicity and Biofilm Formation

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Among the adaptive responses of bacteria to rapid changes in environmental conditions, those of the cell envelope are known to be the most crucial. Therefore, several mechanisms with which bacteria change their cell surface and membranes in the presence of different environmental stresses have been elucidated. Among these mechanisms, the release of outer membrane vesicles (MV) in Gram-negative bacteria has attracted particular research interest because of its involvement in pathogenic processes, such as that of *Pseudomonas aeruginosa* biofilm formation in cystic fibrosis lungs. In this study, we investigated the role of MV formation as an adaptive response of *Pseudomonas putida* DOT-T1E to several environmental stress factors and correlated it to the formation of biofilms. In the presence of toxic concentrations of long-chain alcohols, under osmotic stress caused by NaCl, in the presence of EDTA, and after heat shock, cells of this strain released MV within 10 min in the presence of a stressor. The MV formed showed similar size and charge properties, as well as comparable compositions of proteins and fatty acids. MV release caused a significant increase in cell surface hydrophobicity, and an enhanced tendency to form biofilms was demonstrated in this study. Therefore, the release of MV as a stress response could be put in a physiological context.

The adaptation of bacteria to a rapid change of environmental conditions is a basic survival strategy. The bacterial membrane especially, as a complex interface with the environment, is very sensitive to stress. Therefore, several mechanisms have evolved to stabilize membrane viscosity, e.g., changes in the membrane fatty acid content (14, 17, 19). Due to the fact that Gram-negative bacteria have an outer and an inner membrane with different compositions and important biological functions, both membranes have to remain functional even under changing conditions. As of now, mechanisms affecting the inner membrane have been well studied (34, 35, 42), but the outer membrane and cell surface have received little consideration (5, 11, 31).

The best-investigated mechanism of Gram-negative bacteria, which is related to a change of the outer membrane, is the formation of outer membrane vesicles (MV) (24). MV were often associated with the human pathogen *Pseudomonas aeruginosa* that releases virulence factors with MV to attack the human lung epithelium (20). Since its discovery, this mechanism has been found in many Gram-negative bacteria, and it has become clear that the process of MV formation is highly conserved.

MV are connected to interspecies communication (30), the delivery of proteins, toxins, and DNA (30). Furthermore, the formation of toluene-containing MV was discussed as an adaptation mechanism to transport toxic compounds away from the cells (22). In addition, an important function of MV release is their involvement in biofilm formation (3). Bacteria growing in biofilms are known to have considerable advantages over the planktonic lifestyle. Furthermore, bacteria living in biofilms or microcolonies are significantly more tolerant of antibiotics, biocides, and other forms of environmental stress (3, 5, 15).

The close cell-cell contact also facilitates horizontal gene transfer and sharing of metabolic by-products within the biofilm community (3, 6, 25).

Although tremendous work has been carried out to elucidate the cellular mechanisms leading to the release of MV, there is still not much known about the regulation of this process. Previous research on *P. aeruginosa* has shown that the conversion of 2-heptyl-4-quinolone (HHQ) into 2-heptyl-3-hydroxy-4-quinolone, the so-called *Pseudomonas* quinoline signal (PQS), by the monooxygenase PqsH increases the release of MV (7, 37). However, neither the complete physiological role of MV nor the regulation of their formation has yet been described.

In this study, we investigated the role and physiological function of MV formation in *P. putida* in the presence of rapidly changing environmental stress conditions. The released MV were analyzed with regard to size, surface charge, protein content, and fatty acid composition. In addition, we demonstrated that cells which released MV become more hydrophobic, thus enhancing their ability to form biofilms as a protective mechanism against stress.
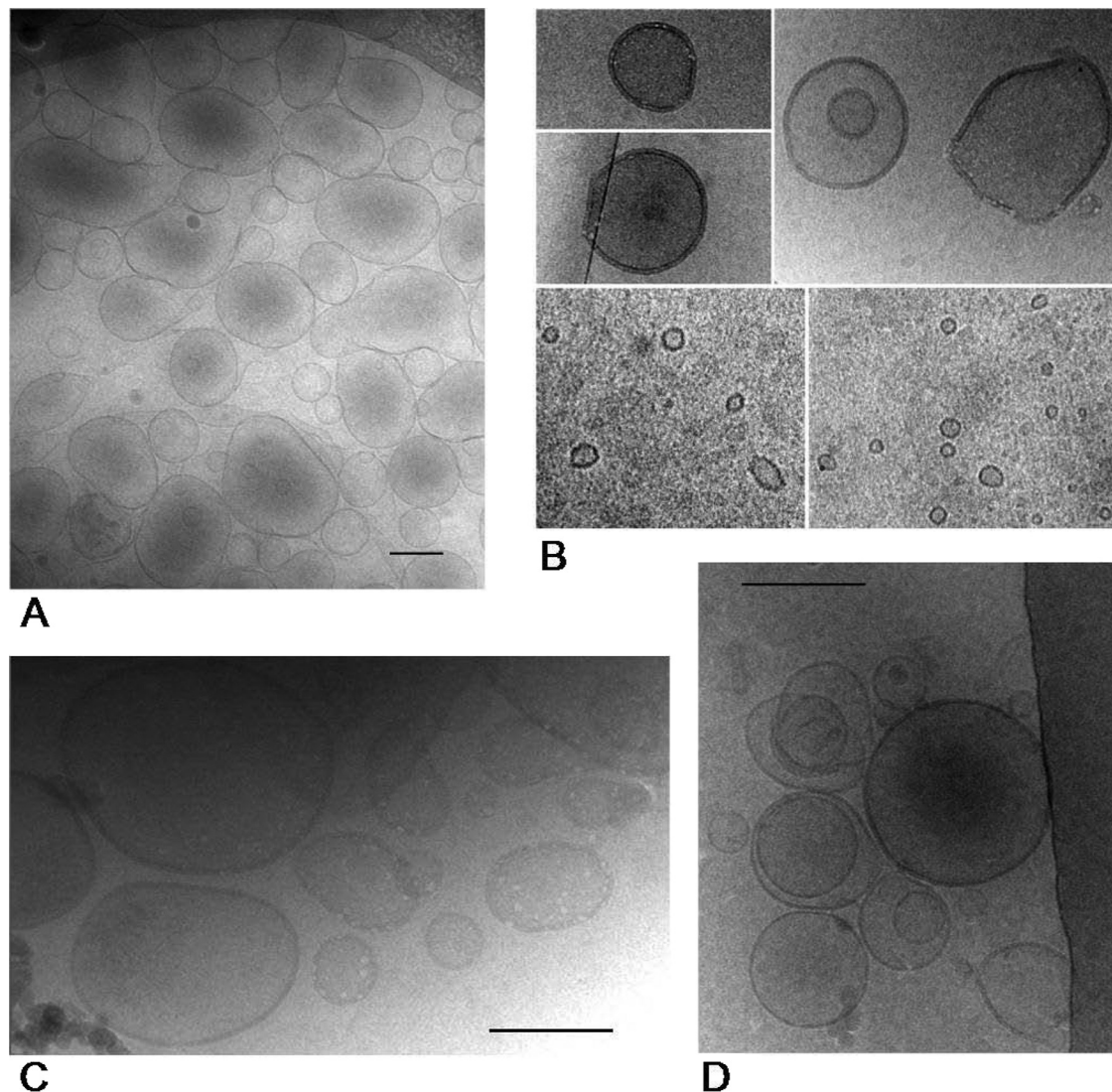
FIG 1 Representative cryo-transmission electron microscopy images of MV formed by *P. putida* DOT-T1E after incubation with 1-octanol (A), NaCl (B), heat shock (C), and EDTA (D). Bars, 200 nm.

MATERIALS AND METHODS

Chemicals and culture conditions. All chemicals were reagent grade and obtained from commercial sources. *P. putida* DOT-T1E (CECT 5312) (33) was cultivated in a mineral medium as described by Hartmans et al. (12), with 4 g/liter disodium succinate as both carbon and energy source. Cells were grown to exponential growth phase in 50-ml shake cultures at 30°C in a horizontally shaking water bath at 180 rpm. Then, either the stressor was added (1-octanol, 1.25 mM; NaCl, 2 M; or EDTA, 10 mM final concentration) or the temperature was increased. After 1 h of incubation, the cells were harvested by centrifugation for 10 min at $6,000 \times g$. The supernatant was used to isolate membrane vesicles as described below. The pelleted cells were washed and finally resuspended in 10 mM KNO_3 solution for further analysis.

Isolation of MV. Isolation of MV was done as previously described (21). The supernatant was filtered through 0.45-μm-pore-size membranes (Sartorius AG, Goettingen, Germany). Subsequently, MV were harvested by centrifugation at $100,000 \times g$ for 3 h at 4°C (L-90K ultracentrifuge; Beckman Coulter). Pelleted vesicles were resuspended in either 50 mM HEPES buffer or 10 mM KNO_3 for Zetasizer analysis.

Cryo-TEM. For cryo-transmission electron microscopy (cryo-TEM), 1 droplet of the isolated MV was applied to a copper grid covered by holey poly-L-lysine-coated carbon film (Quantifoil R3.5/1; Micro Tools GmbH, Jena, Germany), and excess liquid was blotted automatically for 1 s between two strips of filter paper. Subsequently, the samples were rapidly plunged into liquid ethane (cooled to −180°C) in a cryobox (Carl Zeiss NTS GmbH, Oberkochen, Germany). Excess ethane was removed with a

TABLE 1 Zeta potentials and sizes of MV induced by different stresses[a]

Stressor	Zeta potential (mV)	Size (nm) of peak:	
		1	2
1-Octanol	−31.3 ± 3.7		172.2 ± 17.1
Heat shock	−27.1 ± 6.4		154.6 ± 5.8
NaCl	−13.4 ± 3.6	199.1 ± 45.4	45.7 ± 10.7
EDTA	−28.3 ± 9	116 ± 13.3	27.3 ± 11.9

[a] Values are means standard ± deviations.

TABLE 2 Fatty acid composition of MV and stressed cells

	% FAME present after indicated treatment								
	MV				Cells				
Fatty acid	1-Octanol	55°C	NaCl	EDTA	Control	1-Octanol	55°C	NaCl	EDTA
16:0	35.8	36.8	39.7	40.1	31.4	35.2	38.1	32.2	29.4
16:1 Δ9*trans*	4.8	0.3	0.0	0.0	1.2	8.4	2.3	5.6	0.0
16:1 Δ9*cis*	12.5	17.4	10.7	14.3	20.5	13.4	18.6	17.1	20.9
18:0	9.1	7.0	16.7	11.4	1.1	2.0	2.1	1.4	1.2
18:1 Δ11*trans*	2.5	0.1	0.0	0.0	0.2	3.8	0.3	2.9	0.0
18:1 Δ11*cis*	32.2	38.4	32.9	34.2	45.6	36.7	39.0	40.8	47.8
DoS[a]	0.8	0.8	1.3	1.1	0.6	0.6	0.7	0.5	0.4

[a] DoS, degree of saturation.

piece of filter paper. The samples were transferred with a cryo-transfer unit (Gatan 626-DH) into the precooled cryo-electron microscope (Philips CM 120) operated at 120 kV and viewed under low-dose conditions. The images were recorded with a 1-k charge-coupled-device (CCD) camera (FastScan F114; TVIPS, Gauting, Germany).

Characterization of the isolated MV. The electrophoretic mobility (μ) and the average size of MV suspensions in 10 mM KNO$_3$ at pH 6.2 were determined by dynamic light scattering using a Zetasizer (Zetasizer Nano ZS; Malvern Instruments Ltd., Malvern, Worcestershire, United Kingdom) at 150 mV. The zeta potential (ζ), as an indirect measure of surface charge of the MV, was approximated from the electrophoretic mobility according to the method of Helmholtz and Smoluchowski (18).

To characterize the fatty acid profile, extraction and methylation of the fatty acids was done as described before (16). The gas chromatography was executed under the following conditions using a gas chromatograph (6890N; Agilent Technologies, Böblingen, Germany): the GC CP-Sil 88 column (Agilent Technologies) was held at 40°C for 2 min, heated at 8°C per min up to 220°C, and held at that temperature for 5 min. The carrier gas used was He at a flow rate of 2 ml min^{-1}. Fatty acid methyl esters were identified by comparing their retention times to standards.

In order to discover the proteins contained in the isolated MV, equal amounts of supernatant were analyzed by SDS-PAGE. After Coomassie brilliant blue staining, MV protein bands were excised and inserted into microcentrifuge tubes. Then, the bands were destained and digested proteolytically overnight at 37°C using trypsin. The identification of the purified peptides was carried out as reported by Bastida et al. (1) using a UPLC-linear trap quadrupole (LTQ) Orbitrap tandem mass spectrometer (MS-MS), MASCOT (Matrix Science) as the search engine, and bacterial sequences in the NCBI nr (nonredundant) peptide sequence database as the criteria for taxonomy.

Characterization of bacterial cell surface properties. The physicochemical cell surface properties of bacteria were investigated using standard methods as described by others (46). The water contact angle was used to determine the cell surface hydrophobicity, and the zeta potential to characterize the surface charge of MV.

Biofilm formation assay. The quantification of biofilm formation was performed according to O'Toole and Kolter (32). Samples of 1 ml of the stressed cells were adjusted to an optical density at 560 nm (OD$_{560}$) of 0.05 and incubated on polystyrene microtiter plates (Primaria multiwell; VWR International GmbH, Darmstadt, Germany) at 28°C for 18 h. Then, 250 μl of a 0.1% solution of crystal violet (CV) was added and the plates were incubated for additional 30 min at room temperature. Subsequently, the supernatant was removed and each well was washed three times with distilled water. After the plates were dried, 1 ml of ethanol was added twice to remove the bound CV and the absorption was determined at 590 nm.

RESULTS

Characterization of the released MV. In order to investigate the influence of different stressors on the formation of MV, cells of *P. putida* were pretreated with 1-octanol, heat shock, NaCl, or
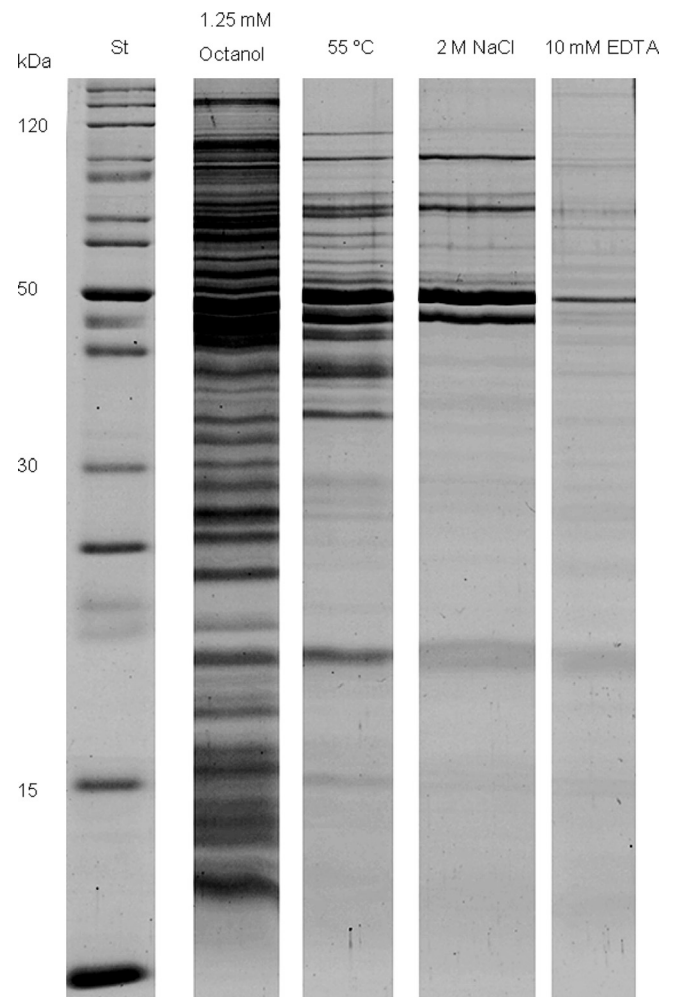

FIG 2 Comparative overview of SDS-PAGE results of the isolated MV. Lane 1, standard; lane 2, 1-octanol-induced MV; lane 3, heat shock-induced MV; lane 4, NaCl-induced MV; lane 5, EDTA-induced MV.

TABLE 3 Proteins identified in MV released from *P. putida* DOT-T1E after different treatments

Protein (source of homologous protein)	NCBI sequence no.	Molecular mass (kDa)	Probability scores after indicated treatment[a]
TonB-dependent receptor (*Pseudomonas stutzeri* A1501)	gi148548751	88.8	NaCl (206.3), 1-oct (55.7), HS (106.6), EDTA (183.1)
Ferripyoverdine receptor (*Pseudomonas aeruginosa*)	gi170282622	85.1	NaCl (243), 1-oct (179.8), HS (107.8), EDTA (72.6)
Elongation factor G (*Pseudomonas mendocina* ymp)	gi146308926	78.8	NaCl (89.4), 1-oct (632.8), HS (391.1)
Outer membrane protein Opr86 (*Pseudomonas aeruginosa* PAb1)	gi296387845	72.9	NaCl (158.9), 1-oct (91), HS (67.6), EDTA (134)
Putative lipoprotein (*Pseudomonas fluorescens* Pf-5)	gi152987388	69.6	NaCl (60.9), 1-oct (66.3), HS (74.7), EDTA (111.5)
Chain A, quinoprotein ethanol dehydrogenase (*Pseudomonas aeruginosa*)	gi10120672	64,0	NaCl (152.8), 1-oct (768.1), HS (461.4), EDTA (375.2)
CopA family copper resistance protein (*Pseudomonas aeruginosa* 152504)	gi313107602	61.8	HS (62.3), EDTA (214.3)
Chaperonin GroEL (*Pseudomonas mendocina* NK-01)	gi152986611	57.0	NaCl (165.9), 1-oct (1168), HS (127.9), EDTA (265.4)
OprD family porin (*Pseudomonas entomophila* L48)	gi104781455	49.4	NaCl (109.8), 1-oct (136.3), HS (131.3), EDTA (99.7)
Putative outer membrane protein precursor (*Pseudomonas aeruginosa* UCBPP-PA14)	gi116049240	45.5	NaCl (136.7), HS (77.4), EDTA (97.7)
OprE3 (*Pseudomonas aeruginosa*)	gi107102299	44.8	NaCl (171.3), 1-oct (72.7), EDTA (135.5)
Elongation factor Tu (*Pseudomonas putida* KT2440)	gi26987181	43.5	1-oct (239.1), HS (161.4), EDTA (136.7)
Bmp family protein (*Pseudomonas syringae* pv. actinidiae strain M302091)	gi32455881	38.7	HS (103.6), EDTA (79.3)
Extracellular solute-binding protein (*Pseudomonas mendocina* ymp)	gi146308575	36.7	NaCl (141.6), EDTA (136.7)
OprF (*Pseudomonas* sp. MFY72)	gi37704628	34.5	NaCl (113.1), 1-oct (122.7), HS (161.3), EDTA (133.5)
Periplasmic binding protein, putative (*Pseudomonas putida* KT2440)	gi26990659	33.2	1-oct (94), EDTA (75.6)
Small protease (*Pseudomonas aeruginosa*)	gi54072636	20.8	HS (105.9), EDTA (95)
Outer membrane protein OmpH (*Pseudomonas fluorescens* WH6)	gi152985326	19.1	HS (91.6), EDTA (107.4)
Peptidoglycan-associated lipoprotein (*Pseudomonas fluorescens* Pf-5)	gi70732082	17.7	NaCl (168.5), 1-oct (117.1), HS (123.8), EDTA (150.6)
Lipopolysaccharide transport periplasmic protein LptA (*Pseudomonas fulva* 12-X)	gi107099955	17.0	NaCl (59.7), 1-oct (62.2), HS (149.3)
Riboflavin synthase subunit beta (*Pseudomonas mendocina* ymp)	gi146308868	16.5	NaCl (76.5), 1-oct (84.2), HS (89.6), EDTA (79.2)
OprL (*Pseudomonas fluorescens*)	gi259090537	16,5	NaCl (78.7), 1-oct (131.7)
50S ribosomal protein L22 (*Pseudomonas aeruginosa* PAO1)	gi15599454	11.9	NaCl (93.7), 1-oct (205), HS (121), EDTA (240.1)

[a] NaCl, 2 M; HS, heat shock (55°C); 1-oct, 1.25 mM 1-octanol; EDTA, 10 mM.

EDTA. Afterwards, the vesicles formed were isolated and analyzed. By cryo-TEM and dynamic light scattering, we could show that different conditions resulted in different MV structures. In contrast to that, no or very few vesicles were detected in the supernatant of nonstressed cells. Figure 1 shows cryo-TEM images of MV isolated from the supernatant. While the vesicles isolated after treatment with 1-octanol or heat shock were similar in size, vesicles isolated after incubation with sodium chloride or EDTA appeared to be smaller (Fig. 1). Due to the limitations of cryo-TEM preparation and to get a partially oriented separation of the MV sizes, the MV were analyzed by dynamic light scattering using a Zetasizer in order to obtain the charge and a better size distribution. Regarding the size, one peak in the range of 110 nm up to 200 nm was observed for all conditions. In addition, after the treatment with EDTA and sodium chloride, another peak appeared in the range of 20 nm to 50 nm. In the case of the zeta potential, sodium chloride-induced MV had a considerably higher value than MV exposed to the other conditions (Table 1). Thus, most probably, salt ions were attached to the surface of the vesicles, causing this effect.

Due to the obvious differences of the released MV, their fatty acid and protein compositions were analyzed. The results

of the fatty acid methyl ester (FAME) analysis showed an approximately 5-fold enrichment of stearic acid (18:0) compared to the amount in the cells (Table 2). This enrichment of stearic acid was previously reported for outer MV (44) and is herewith confirmed. All in all, the degree of saturation of the MV was also increased and was even higher for sodium chloride- and EDTA-induced MV. The lesser degree of saturation for MV released after 1-octanol treatment or heat shock can be partially explained by cell lysis releasing membrane lipids into the medium.

MV were also analyzed for their protein content. SDS-PAGE (Fig. 2) showed the protein profiles of the vesicles induced under different conditions. One consideration was that treatment with 1-octanol causes enhanced membrane permeability, which could lead to an easier release of cytoplasmic proteins. Due to the high sensitivity of mass spectrometry, the resulting data had to be analyzed very carefully. Therefore, only proteins appearing under more than one condition are listed in Table 3. It is noticeable that mostly outer membrane proteins were detected after every treatment.

Rapid changes in cell surface properties in response to different stressors. Since the release of MV is known to be related to
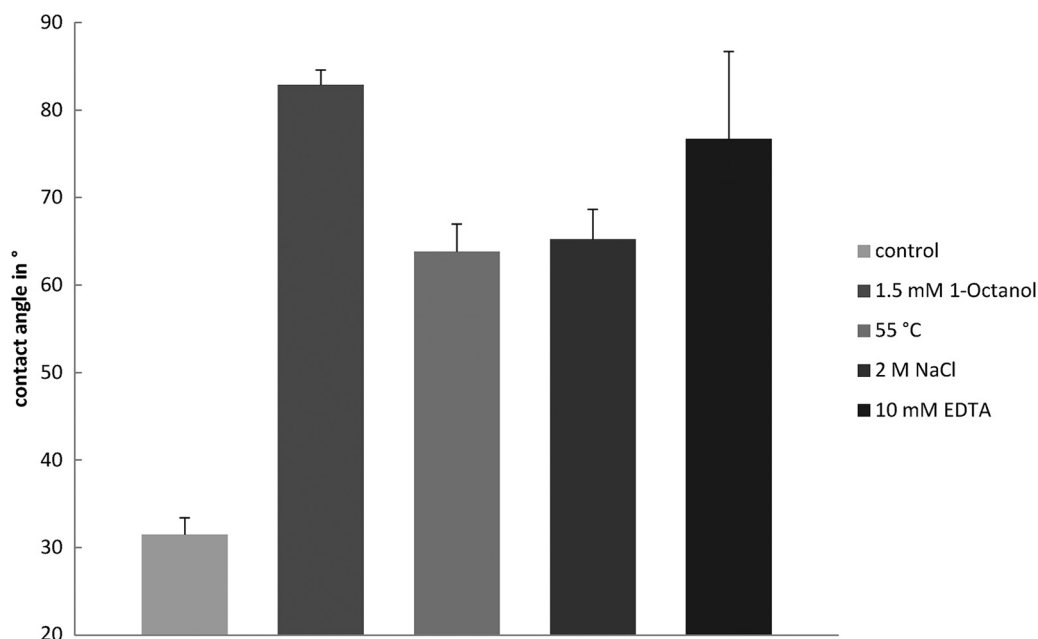
FIG 3 Effects of 1-octanol (A), heat shock (B), NaCl (C), and EDTA (D) on water contact angles of growing cells of *P. putida* DOT-T1E. Error bars show standard deviations.

a change in cell surface properties of *P. putida* (2), different conditions were tested for a similar response (Fig. 3). Interestingly, treatment of the cells with EDTA or high concentrations of sodium chloride caused results analogous to those induced by the addition of 1-octanol or heat shock (55°C). For all conditions, the kinetics showed the same progress, indicating that the process was almost finished after the cells had been incubated for 10 min in the presence of a stressor (see Fig. S1 in the supplemental material). The same reaction was observed when the cells were pretreated with 1 mM chloramphenicol, demonstrating that this response is independent of *de novo* protein biosynthesis. In contrast, neither 2,4-dichlorophenol nor 2,4-dinitrophenol caused any notable effect (data not shown).

Biofilm formation of stressed cells. In order to investigate possible advantages of the release of MV, the ability of pretreated cells to form biofilms was investigated. It was observed that the growing cells did not cover the polystyrene surface homogenously but built more complex structures (Fig. 4). This is in agreement with previous studies (25).

The biofilm formation was also quantified. Because every treatment caused the release of MV, as well as an inhibition of growth rates, HgCl$_2$-treated cells were used as a control (2). The addition of 0.1 mM HgCl$_2$ slightly reduced the growth rate without changing the outer membrane properties. In comparison to this control, all other samples showed an increased capability of forming biofilms (Fig. 5), showing that the release of MV as a stress response leads to an increased hydrophobicity and, in consequence, to enhanced biofilm formation.

DISCUSSION

The central finding of the present work is the release of MV as a reaction of the cells to several stress factors. This release of MV led to an increase in the cell surface hydrophobicity, which led to a

strong tendency to form biofilms (3, 11, 25). This physiological sequence has never been described before.

In order to improve the knowledge about this stress response mechanism, the MV released were analyzed. Besides those of the usual size, with about a 150-nm radius, smaller vesicles occurred after treatment with osmotically effective concentrations of sodium chloride or EDTA. Both compounds probably disturb the interaction of calcium ions with lipopolysaccharide (LPS) by either complex formation (EDTA) or display of the calcium ions from the negative charged residues of the LPS. Consequently, this changed calcium-LPS composition could affect a potential mechanism involved in the formation of MV and leading to smaller vesicles. The zeta potential of the released MV correlates well with that of the cells (2). Because the released MV and the cell surface of nonstressed cells have similar charges, comparable surface compositions can be assumed. It was also previously shown that the zeta potential of *P. putida* changes after organic solvent stress (31).

The analysis of the fatty acid components demonstrates that the MV contained mainly C$_{16:0}$ and C$_{18:1\Delta11cis}$. However, a considerable enrichment of stearic acid (18:0) was found in the MV compared to the amount in the cell membranes. This is consistent with other studies (44) and indicates that the released vesicles are formed from the outer membrane of *P. putida*.

The proteomic data of the isolated MV likewise illustrate a composition similar to the outer membrane of *P. putida*. Generally, the different outer membrane proteins identified were consistent with the results of other studies (4), e.g., OprD and OprF. Several unusual proteins were identified in vesicles after every treatment. Chain A of the quinoprotein ethanol dehydrogenase is known as a redox active enzyme using a special cofactor, pyrroloquinoline quinine (PQQ). It is supposed that all enzymes belonging to this class are located in the periplasm (9).
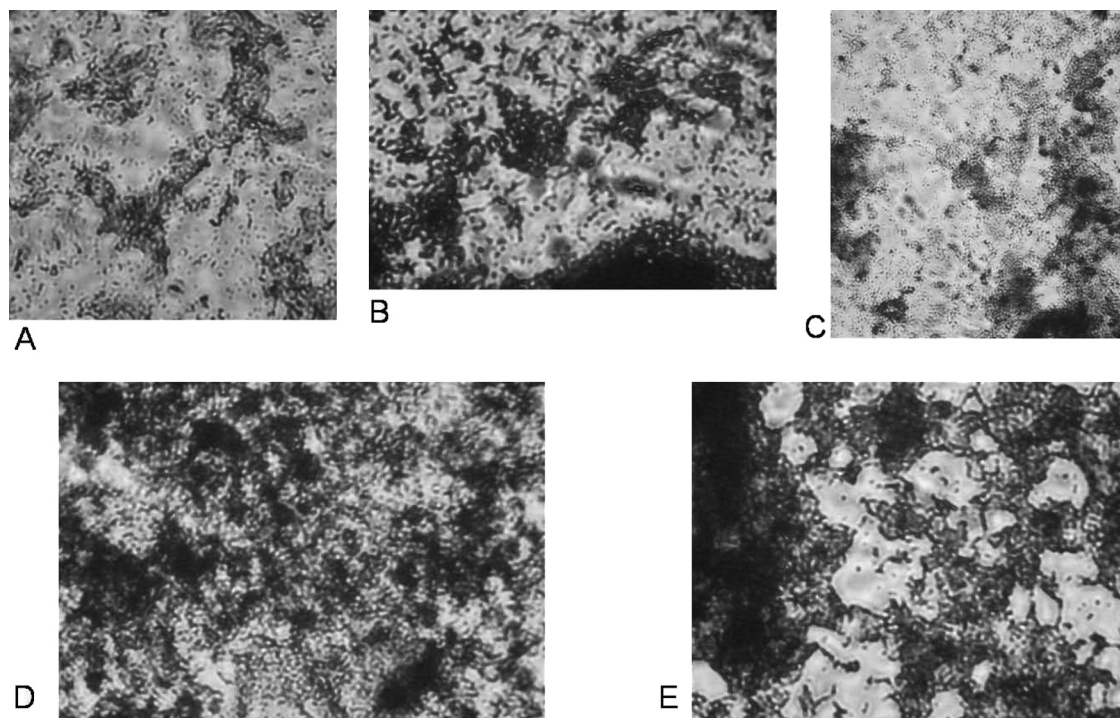
FIG 4 Microscopic images of biofilms formed by *P. putida* on polystyrene microtiter wells after incubation with HgCl$_2$ (A), 1-octanol (B), heat shock (C), NaCl (D), and EDTA (E).

In addition, other periplasmatic proteins were abundant in the MV. Recently, a sorting mechanism to pack proteins into MV was proposed (13). Interestingly, ribosomal proteins and elongation factors occur in the proteome of MV. Either these proteins are released by the cells after the exposure to the stressor due to an enhanced permeability of the membrane, including partial cell lysis, or they are specifically included in MV. The latter hypothesis is supported by the fact that *P. aeruginosa*-derived vesicles contain nucleic acids (23, 40).

The kinetics of MV release observed in our study indicates that it takes around 10 min until this process is completed. Therefore, transcriptional induction can be excluded. Instead,

FIG 5 Quantification of biofilm formation of *P. putida* after incubation with different stressors. Error bars show standard deviations.

the system is most probably continuously expressed and can be allosterically regulated simply by activating an already existing cellular process. Such an assumption is in agreement with the results of other studies of both organisms, *P. putida* and *P. aeruginosa* (2, 26).

Since it was noticed that membrane vesicles play an important role in bacterial life, several mechanisms for their formation were proposed (29, 30). Considering our knowledge of the membrane as a mixture of so-called lipid rafts (10), the different types of the LPS layer might be organized in this way. Furthermore, it is assumed that lipid rafts fulfill an important function in signaling and vesicle formation in eukaryotic cells (41). It was shown that the PQS molecule interacts specifically with outer membrane lipids (28, 38). Thus, an enrichment of PQS or another signaling molecule in the outer membrane could potentially rearrange the lipid rafts, leading to a local accumulation of long-chain LPS (28, 36). This fractional enrichment could cause the formation of blebs on the outer site of the outer membrane and can be proposed as the initial step of the formation of MV (30). So far, the precursor HHQ but not PQS has been detected in *P. putida* (7, 8). Thus, although the enzyme responsible for PQS formation from HHQ in *P. aeruginosa* is not yet described in *P. putida*, it seems reasonable that PQS or a very similar quorum-sensing molecule is involved in the release of MV in *P. putida* (7, 8). This hypothesis is supported by the fact that exogenous PQS enhances MV production in *P. putida* (43).

The biological importance of the formation of MV is well described (39, 45). Very recently, Manning and Kuehn (27) demonstrated that *E. coli* releases MV as a fast response to envelope stress, leading to increased survival of the bacteria. Furthermore, the
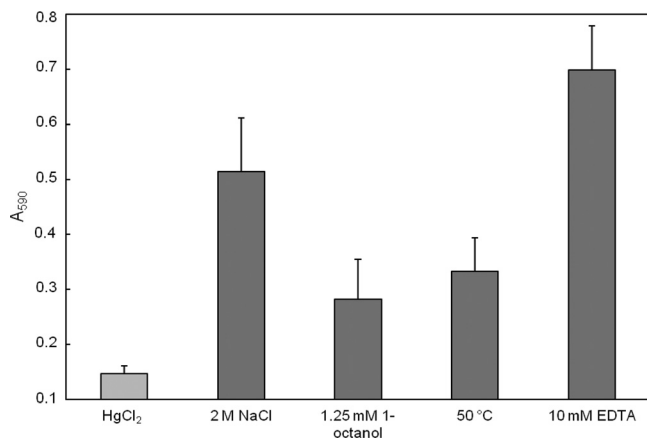
Stressor

(heat shock, n-alkanols, EDTA, NaCl, membrane intercalating antibiotics)

Synthesis of a signalling molecule that interacts with LPS; so far not yet identified for *P. putida*

Release of membrane vesicles

Increase in cell surface hydrophobicity
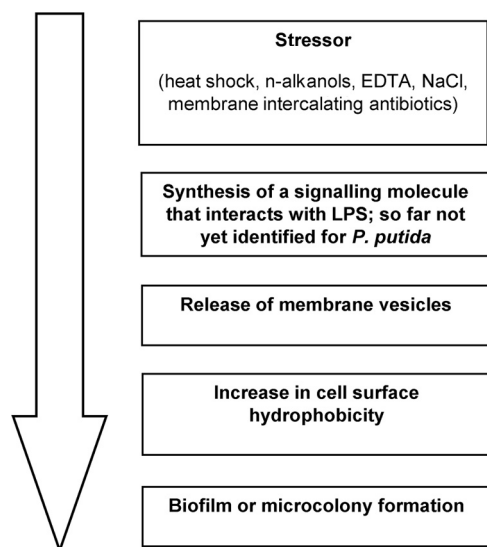
Biofilm or microcolony formation

FIG 6 Possible sequence of biofilm formation in *P. putida* after exposure to a stressor.

connection to biofilm formation was assumed before (3) and appears to be confirmed by the results of this study.

In conclusion, this study indicates that cells of *P. putida* sensing stress release MV, which leads directly to a highly hydrophobic cell surface. Consequently, they attach easily to each other, as well as to surfaces. This mechanism is probably conserved in most Gram-negative bacteria and involved in biofilm formation as an important mechanism to survive environmental stress conditions (Fig. 6).

ACKNOWLEDGMENTS

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