DNA-containing membrane vesicles of
Pseudomonas aeruginosa PAO1 and
their genetic transformation potential

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Natural membrane vesicles (n-MVs) produced by Pseudomonas aeruginosa PAO1 and PAO1 carrying plasmid pAK1900 (p-MVs) were purified and analysed for DNA content. The MVs were isolated by a procedure designed to ensure no cellular contamination from the parent MV-producing cells. Fluorometry analysis revealed that p-MVs were associated with 7-80 ng DNA (20 μg MV protein)⁻¹. PCR analysis using specific primers for pAK1900 sequences and a chromosomal target, oprL, indicated that only plasmid DNA was contained within the lumen of p-MVs after exogenous DNA was digested by DNase. MVs have previously been shown to be capable of fusing into the outer membrane (OM) of PAO1 and Escherichia coli DH5α. Accordingly, p-MVs should deliver the plasmid into the periplasm, where it would only have to by-pass the plasma membrane (PM) for effective transformation. It was speculated that p-MVs should increase transformation efficiency but the data suggested otherwise. p-MVs did not transform PAO1 nor DH5α under a variety of transforming conditions. To characterize p-MVs and to ensure that membrane-encapsulated pAK1900 was not derived from a small proportion of lysed cells within the culture and bound by PM instead of OM, which typically forms n-MVs, the physical and ultrastructural differences between n- and p-MVs were determined. Cryo-transmission electron microscopy (cryo-TEM) revealed that n-MVs and p-MVs closely resembled isolated OM. Buoyant density measurements using isopycnic sucrose gradients on isolated PM, OM, n- and p-MVs demonstrated that isolated OM and n-MVs both fractionated into two bands (ρ = 1·240 and 1·260 g ml⁻¹). p-MVs also produced two bands but at two different densities (ρ = 1·250 and 1·265 g ml⁻¹) which may be attributed to the presence of DNA. SDS-PAGE showed that p-MVs possessed most major OM proteins and also contained 43·70 nmol 3-deoxy-D-manno-octulosonic acid (KDO) (mg protein)⁻¹ as an LPS marker. The amount of NADH oxidase activity, a PM enzyme, in the p-MVs was barely detectable. These data strongly suggest that p-MVs are OM-based, with little if any PM material associated with them. The possibility of whether exogenous plasmid DNA could enter n-MVs once the vesicles had departed from cells was also tested; surprisingly, a small amount of DNA could. Accordingly, the data suggest that DNA can be taken up by MVs using two separate routes: (1) via a periplasmic route and (2) via an extracellular, exogenous route.

INTRODUCTION

Pseudomonas aeruginosa PAO1 naturally produces membrane vesicles (n-MVs) which are liberated from its outer surface (Beveridge, 1999). These bilayered spheres contain phospholipid, LPS, outer-membrane protein (OMP), periplasmic material and, in some cases, DNA (Kadurugamuwa & Beveridge, 1995, 1997; Beveridge & Kadurugamuwa, 1996; Li et al., 1996). n-MVs have been observed in laboratory-grown cultures and in natural environments such as freshwater, soil and biofilms (Forsberg et al., 1981; Beveridge et al., 1997). Since the production of MVs is a common phenomenon, since they represent a significant metabolic expense to cells and since they are produced by a multitude of Gram-negative bacteria (Li et al., 1998), they must serve a significant purpose. Some possible roles have been suggested, such as predation (Kadurugamuwa & Beveridge, 1996; Kadurugamuwa et al., 1998; Li et al., 1998; MacDonald & Beveridge, 2002), enhanced virulence

Abbreviations: KDO, 3-deoxy-D-manno-octulosonic acid (3-deoxy-D-manno-2-octulosonic acid); MVs, membrane vesicles; n-MVs, natural membrane vesicles; OM, outer membrane; PM, plasma membrane; p-MVs, MVs from PAO1/pAK1900; TEM, transmission electron microscopy.
It is remarkable that DNA is packaged into MVs of *Escherichia*, *Haemophilus*, *Neisseria* and *Pseudomonas* species (Kahn et al., 1982, 1983; Dorward & Garon, 1989; Dorward et al., 1989; Kadurugamuwa & Beveridge, 1995; Kolling & Matthews, 1999; Yaron et al., 2000), since the process of encapsulation must be complex. Studies on certain Gram-negative bacteria suggest that not only are plasmids packaged but also chromosomal DNA and even bacteriophage DNA. This DNA somehow migrates from the cytoplasm, through the plasma membrane (PM) to the periplasm and (along with other periplasmic materials; Kadurugamuwa & Beveridge, 1995) is encapsulated within an MV. Once liberated from the cell, foreign autolysins (Li et al., 1996, 1998; Kadurugamuwa et al., 1998; MacDonald & Beveridge, 2002) or β-lactamases (Ciofu et al., 2000) can be delivered to other bacteria. There are certain advantages for DNA to encapsulate itself in MVs. DNA would be protected from exonucleases. Furthermore, MVs should also enhance the efficiency of DNA delivery and uptake into a recipient cell as they fuse into the outer membrane (OM). Because naturally competent bacteria are genetically programmed to permit the efficient uptake of macromolecular DNA (Dubnau, 1999) and non-competent bacteria lack such a mechanism, MVs could possibly overcome such genetic barriers.

To date, DNA within MVs has been identified from competent *Neisseria gonorrhoeae* and *Haemophilus influenzae* and non-competent *P. aeruginosa* and *Escherichia coli* O157:H7. MVs of *N. gonorrhoeae* contained linear and chromosomal DNA including 4·2 and 7·1 kb plasmids (one of which carried a penicillin-resistance gene) along with small amounts of RNA (Dorward et al., 1989). Following incubation of these MVs with penicillin-sensitive gonococci, transformants were recovered in the presence of the antibiotic. These data imply that MV-mediated plasmid transfer occurs among gonococci and possibly with other cohabiting bacteria. Incubation of MVs from *E. coli* O157:H7, which carried ampicillin resistance (AmpR) and green fluorescent protein genes on a plasmid (pGFP), with *E. coli* lacking this plasmid resulted in fluorescent transformants, which also suggests that *E. coli* MVs act as vectors of DNA transport (Yaron et al., 2000).

DNA has also been found associated with MVs isolated from *P. aeruginosa*, but little has been done to characterize this system and to determine its transformation potential. Our study not only investigates the DNA content of MVs from PAO1/pPK1900 (p-MVs) and whether these MVs can transform PAO1 and *E. coli* DH5α, it also determines physical differences such as buoyant density and ultrastructure between p-MVs and n-MVs. Because it is unclear how cellular DNA, a component that normally resides in the cytoplasm, is incorporated within these periplasm–containing MVs, we endeavoured to clarify the origin of this DNA. It is possible that DNA could be inserted into MVs before the vesicles leave the bacterium. Here the encompassing material could be PM, OM, or both (i.e. as a double bilayered structure). It is also possible that exogenous DNA could somehow enter the MV after the vesicle has left the cell.

Because a stringent MV isolation procedure was necessary to ensure no cellular contamination of cells from the parent strain, our previous protocol to isolate MVs (Kadurugamuwa & Beveridge, 1995) required more rigorous precautions. Accordingly, this work describes a system to prepare MVs that is completely free of donor cells to ensure that transformants obtained following MV transformation experiments are due to DNA transfer via MVs and not due to a contaminating source.

**METHODS**

**Bacterial strains and culture conditions.** *P. aeruginosa* PAO1 serotype O5, PAO1/pPK1900 and *E. coli* DH5α were grown in Trypticase soy broth (TSB) (BBL Laboratories) to late exponential phase (~108 c.f.u. ml⁻¹) on an orbital shaker (125 r.p.m.) at 37 °C. To select for the presence of PAO1/pPK1900, 500 µg carbenicillin ml⁻¹ (Sigma) was added to the culture medium. pAK1900 is an *E. coli*–*P. aeruginosa* shuttle cloning vector and encodes ampicillin and carbenicillin resistance (Jansons et al., 1994).

**Isolation of MVs containing periplasmic DNA.** PAO1 and PAO1/pPK1900 MVs were isolated by the protocol originally designed by Kadurugamuwa & Beveridge (1995). Exponentially growing bacteria were centrifuged at 6000 g, the cell pellet discarded, and the supernatant sequentially filtered three times through 0·45 and 0·22 µm cellulose acetate membranes (Osmonics) to remove any remaining cells. Ultracentrifuge tubes were pre-soaked in a sterilizing agent (1/100 dilution of Sani Rinse; Wil-Don) for 30 min and rinsed three times in sterilized nanopure water to remove residual detergent. MVs were recovered from the resulting filtrates by centrifugation at 150 000 g for 1·5 h at 5 °C in a Ti45 rotor (Beckman Instruments). The supernatant was carefully aspirated from the tubes and the vesicle pellet was resuspended with 50 mM HEPES buffer (pH 6·8; Sigma) and centrifuged at 16 000 g in a bench-top centrifuge for 30 min to concentrate the MV pellet. The pellet was washed with HEPES and centrifuged at 16 000 g for 30 min. The MV protein content was determined using a Micro BCA protein assay reagent kit (Pierce). The purity of the MV sample was examined by transmission electron microscopy (TEM) and by checking for growth of any remaining parent cells on Luria–Bertani (LB) agar. Aliquots representing 1/10 of each MV preparation (50 µg total protein) were plated and incubated overnight at 37 °C to observe growth. If no colonies were observed after this period and, in addition, if no cells were seen by TEM, the MV preparation was stated to be free of cells and thought to be sterile.

**Fluorometric quantification of DNA.** Surface-associated DNA and DNA contained within MVs was quantified by the PicoGreen dye assay (Molecular Probes). A 20 µg protein sample from n-MVs or p-MVs was lysed with GE reagent [5 M guanidinium thiocyanate, 100 mM EDTA, 0·5 % (v/v) Sarkosyl] to release DNA from MVs. Experiments were also performed with MVs that were pre-treated with 50 µg ml⁻¹ pancreatic DNase I (Sigma) and 10 mM MgCl₂ (1 h at 37 °C) to digest DNA possibly bound to the outer surface of MVs, after which the vesicles were washed and then lysed with GE reagent.
reagent. Samples were further processed according to the manufacturer’s instructions and fluorescence was measured in a Hitachi F-2000 fluorescence spectrophotometer.

**PCR.** PCR was performed in a total volume of 100 μl containing 150 pmol of each primer, 0.2 mM of each deoxynucleoside triphosphate, 1× PCR buffer (Roche), 1.5 mM MgSO4, nucleotide-free water and 2-5 units Pwo polymerase (Roche). DNA primers used for the amplification of the β-galactosidase (lac) gene on pAK1900 were pAKF (5’-GACCATGATTCGCGAGCT-3’) and pAKR (5’-TTTGGGCTGCAGGTGCCG-3’), to amplify a product of 475 bp. Primers designed for the chromosome encoded pAK1900 (~3 ng) were used separately as positive controls. PCR amplification consisted of a 3 min hot start of 94°C followed by 15 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C and continued with an additional 10 cycles of 30 s at 94°C, 30 s at 55°C and 1 min plus 5 s per additional cycle at 72°C. The reaction was completed with an extension step of 5 min at 72°C. PCRs were carried out in a Minicycler PCR system (MJ Research). PCR products were separated by electrophoresis on 0-8% agarose gels containing ethidium bromide and visualized by UV transillumination.

**Transformation experiments.** Transformation experiments were adapted from Yaron et al. (2000) and were performed using p-MVs with either PAO1 or E. coli DH5α as the recipient. Recipient cells were grown to exponential phase and resuspended in fresh LB, SOC (a rich medium that contains concentrations of glucose and magnesium that maximize transformation efficiency) or HEPES, to a concentration of 10^7–10^9 cells ml^{-1}. An MV sample (100 μg DNA ml^{-1}, 50 μg DNase ml^{-1}, the cell suspension (100 μl) and 800 μl of medium (either LB, SOC or HEPES) were combined and incubated at 37°C statically for 1 h, followed by 2 h with shaking. A volume of 10 ml of LB, SOC or HEPES was added to the samples and incubated for an additional 20 h with shaking. Aliquots (100 μl) of this suspension at 2, 4, 8, 12 and 20 h were plated on LB/carbonicillin and incubated overnight at 37°C. Colonies observed on plates were successful transformants. Experimental controls were n-MVs or 1 μg ml^{-1} of purified pAK1900 DNA, in place of p-MVs, under the same transformation conditions. MVs were spread directly on LB plates, and PAO1 and DH5α were spread on LB and LB/carbonicillin plates.

**Fusion of p-MVs with recipient cells.** Fusion experiments were done in a similar manner to those performed by Kadurugamuwa & Beveridge (1999). An exponentially growing culture of PAO1 in TSB was washed and diluted in PBS (pH 7.4) to produce a bacterial suspension of 1×10^8 c.f.u. ml^{-1}. These cells were mixed 1:1 with a suspension of p-MVs (100 μg protein ml^{-1}) that were pre-labelled with cationized ferritin (an electron-dense probe for TEM) and incubated at 37°C for 30 min. The unbound MVs were removed from the cells by centrifugation (6000 g, 15 min) and the pellet was resuspended in 100 μl PBS. Samples were examined by TEM to determine if the labelled p-MVs adhered to and fused into the recipient cells.

**Cryo-transmission electron microscopy (cryo-TEM).** Thin frozen films of n-MVs, p-MVs and PAO1 OM vesicles were prepared according to the procedure of Adrian et al. (1998). Briefly, a drop of vesicles resuspended in either 50 mM HEPES buffer (pH 6-8; see below) or deionized water (for OM vesicles; see grid) was spread on a holey carbon-coated 600-mesh copper EM grid. The excess liquid was blotted with filter paper and the grid was then instantly plunged into liquid propane cooled by liquid nitrogen. Grids were mounted in a Gatan 626DH cryo-holder and cryo-TEM examinations were carried out at ~170°C in a LEO 912AB microscope, operating at an accelerating voltage of 100 kV. Images were acquired with a defocus between ~1 and ~2 μm (depending on the magnification) using a Proscan HSC2 slow-scan CCD camera and total electron doses per viewing area were less than 1500 electrons nm^{-2}. Bilayer thickness measurements of the digital images were done using the analySIS software. Intact vs broken p-MVs and OM vesicles were counted.

**Isopycnic gradients and determination of buoyant densities (ρ).** OM and PM were purified as described previously (Hancock & Nikaido, 1978; Horstman & Kuehn, 2000) with some modification. Cells from an overnight 1 litre culture of PAO1 were harvested, washed twice in 100 mM HEPES (pH 6-8), resuspended in 20 ml of 100 mM HEPES, 1 mg lysosome ml^{-1}, 50 μg DNase 1 ml^{-1}, 100 μg RNase III-A ml^{-1}, 20% sucrose (w/v) and French pressed twice at 124 MPa. The suspension was centrifuged (1000 g, 10 min) to remove any intact cells. The supernatant was layered on a sucrose cushion [5% and 55% (w/v) sucrose in 30 mM Tris (pH 8-0)] and centrifuged (183 000 g, 3 h) in a SW41 rotor (Beckman). The membranes were removed from the interface and 30 mM Tris (pH 8-0) was added to give a final volume of 2-5 ml. This suspension was loaded on a sucrose step gradient [60, 55, 50, 45, 40, 35% (w/v) sucrose in Tris] and centrifuged to equilibrium (183 000 g, 18 h). The resulting bands were carefully removed by a syringe needle, washed five times with sterile, nanopure water and pelleted (15000 g). p-MVs and n-MVs (25 MV isolation runs each) were also separately loaded onto similar isopycnic sucrose gradients and centrifuged to determine their buoyant densities. Sucrose densities for correlation with resulting bands were determined by refractometry of gradient samples.

**KDO assay.** KDO (3-deoxy-D-manno-octulosonic acid) was determined by the thiobarbituric acid method (Karkhanis et al., 1978) with purified KDO (Sigma) as a standard.

**NADH oxidase assay.** The measurement of NADH oxidase followed the method of Osborn et al. (1972). The rate of decrease in absorbance at 340 nm was measured in a Shimadzu UV-mini 1240 spectrophotometer (Mandel Scientific).

**SDS-PAGE.** A 40 μg sample of protein from isolated OM, PM, n-MVs and p-MVs was solubilized in sample buffer [15-7% (v/v) Tris (pH 6-8), 3-1% SDS, 15-7% (v/v) glycerol, 3-1% (v/v) 2-mercaptoethanol and 2-5% bromophenol blue], heated to 100°C for 10 min and analysed by SDS-PAGE as described previously (Kadurugamuwa & Beveridge, 1995). The gel was subsequently stained with Coomassie Brilliant Blue R-250 (CI 42660) and destained with 5% (v/v) acetic acid (aq.).

**Exogenous DNA encapsulation assays**

**DNase assay.** Two 500 ml TSB flasks were inoculated with PAO1/pAK1900. Flask A represented the control and flask B was supplemented with a final concentration of 100 μg DNAse ml^{-1} (Roche) and 10 mM MgCl2. The flasks were incubated at 37°C (120 r.p.m.) until late-exponential phase and MVs were isolated. The internalization of DNA within NVs in the presence of DNase was determined by PCR. A 10 μl sample of untreated MVs (flask A) and DNase-treated MVs (flask B) was used as template. A 5 μl aliquot of the final ultracentrifuged supernatant (free of cells and MVs) from both flasks was analysed to determine whether extracellular DNA was present. Positive controls were purified pAK1900 and genomic whole-cell DNA. Samples containing DNase were heated at 100°C for 15 min before being analysed by PCR (Tavares & Sellstedt, 2001).

**n-MVs+pAK1900 assay.** n-MVs (500 μg ml^{-1}) were incubated with pAK1900 (1 μg ml^{-1}) for 16 h at 37°C with slight agitation.
Half of this sample was treated with DNase (50 μg ml⁻¹) while the other half was left untreated. The external or internal association of pAK1900 within n-MVs was investigated by PCR. Heat-inactivation of DNase was performed on samples before PCR. As a negative control, the hydrolysis activity of DNase on pAK1900 was checked by incubating 1 μg pAK1900 with 50 μg DNase ml⁻¹ for 1 h at 37°C. DNA encapsulated within n-MVs was quantified by the PicoGreen fluorometric DNA assay.

## RESULTS

### Confirmation that MVs contained intact DNA derived from whole cells

PAO1 was artificially transformed with plasmid pAK1900 so that MVs isolated from this strain could be analysed for the possible incorporation of plasmid DNA. A cell-free MV isolation system was successfully designed (as described in Methods) since no colonies were recovered after incubation on nutrient agar.

Fluorometric quantification of DNA associated with MVs using the PicoGreen reagent ensured ultrasensitive detection of double-stranded DNA with minimal fluorescence contributed by RNA and single-stranded DNA. The results showed 0.31 ng DNA (20 μg MV protein)⁻¹ associated with n-MVs (Table 1), which confirmed previous results of Kadurugamuwa & Beveridge (1995). Most of this DNA was contained within the n-MVs and not surface associated since ~70% of DNA content remained following DNase treatment. p-MVs contained ~25 times more DNA, but only half of this was inside the MVs (Table 1).

### Determination that this internalized DNA was of plasmid origin

PCR was performed to examine the type of DNA within MVs. Primers were designed for oprL (a chromosomal gene) and lac (a plasmid-encoded gene). PCR amplification of oprL (760 bp) showed that chromosomal DNA was only externally associated with n-MVs since no product was produced after DNase treatment of intact MVs (Fig. 1a). This represents a small discrepancy with the fluorometric results for DNase-treated n-MVs, where a small amount of DNA was detected. Because the DNA concentration detected by fluorometry was minimal (100 times less than p-MVs) and could be due to the contribution of small amounts of RNA and hyrolysed DNA, we believe that chromosomal DNA is only surface associated.

Using p-MVs as the template, the amplification of oprL and lac (475 bp) indicated that not only did the p-MVs contain externally associated chromosomal DNA but also internally and externally associated plasmid DNA (Fig. 1b). As with the amplification of oprL, DNase treatment digested surface-associated chromosomal DNA from the MVs.

### Investigating the transformation potential of p-MVs

With the presence of pAK1900 within MVs, the involvement of p-MVs as DNA delivery agents was explored. We expected that DNA within MVs would be protected from exonucleases, thereby increasing the efficiency of DNA delivery and uptake into a recipient cell. Although the internal plasmid DNA was unaffected by DNase treatment, p-MVs did not have the ability to transform PAO1 nor DH5α. Time-course p-MV transformation experiments of

### Table 1. DNA content in MVs derived from PAO1 and PAO1/pAK1900

MVIs were lysed with GES reagent (see Methods).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vesicle type</th>
<th>DNA content [ng (20 μg MV protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>n-MV</td>
<td>0.31 ± 0.17</td>
</tr>
<tr>
<td>PAO1 DNase-treated</td>
<td>n-MV</td>
<td>0.23 ± 0.10</td>
</tr>
<tr>
<td>PAO1/pAK1900</td>
<td>p-MV</td>
<td>7.80 ± 0.41</td>
</tr>
<tr>
<td>PAO1/pAK1900 DNase-treated</td>
<td>p-MV</td>
<td>3.91 ± 0.31</td>
</tr>
</tbody>
</table>

*Means ± SD, n = 3.
Characterization of bacterial vesicles

1–20 h with PAO1 and DH5α as the recipients, in nutrient-rich (LB and SOC) media and under nutrient-limited conditions (HEPES buffer), produced no transformants. To prove that this lack of transformation was not due to the inability of p-MVs to fuse to the recipient strains, MVs were pre-labelled with cationized ferritin (as an electron-dense probe) and then incubated with recipient cells. The recipients became labelled with cationized ferritin, proving that the p-MVs had fused into the OM of the cells (data not shown). The process of p-MV adhesion and fusion mimicked the results of Kadurugamuwa & Beveridge (1999).

The experimental controls confirmed that: (1) n-MVs or purified pAK1900 did not produce transformants, (2) donor MVs did not produce colonies on LB plates, and (3) recipient cells grew on LB plates but did not grow on LB/carbenicillin plates.

**Is the make-up of p-MVs different from n-MVs?**

This study was performed to define the structure and composition of MVs. Thin frozen films observed by cryo-TEM revealed that n-MVs and p-MVs each possessed a single membrane bilayer that was identical in thickness (Table 2) and contrast intensity to that of the PAO1 OM (Fig. 2). Since these preparations were snap-frozen and physically fixed in vitreous ice, they remain in a hydrated state and represent the most authentic natural view of MVs. To distinguish the make-up of this bilayer, i.e. whether it is OM- or PM-based, the densities of the MVs were estimated using isopycnic sucrose density gradients (Table 2). For comparison, the densities of purified PM and OM were determined. Both n- and p-MVs fractionated into two bands but at different densities (Fig. 3): p-MVs at ρ = 1.250 g ml⁻¹ and 1.265 g ml⁻¹ and n-MVs at the same density as the purified OM fractions (ρ = 1.240 and 1.260 g ml⁻¹). The difference in densities between n-MVs and p-MVs may be attributed to the presence of DNA contained within the p-MVs. It is possible that both n- and p-MVs are formulated from discrete regions of the OM possessing different OM densities.

When enzymic markers for OM (i.e. KDO) and PM (i.e. NADH oxidase) were assayed, it was apparent that n- and p-MVs contained only KDO [41.02–43.70 nmol (mg protein)⁻¹] with virtually no NADH oxidase [0.07–0.27 nmol min⁻¹ (mg protein)⁻¹] (Table 2). The protein profiles of OM, PM, n-MVs and p-MVs were compared by SDS-PAGE after gels were stained with Coomassie Brilliant Blue (Fig. 4). The banding patterns of n- and p-MVs were similar, but not identical to the corresponding OM pattern. According to the electrophoretic classification of OMPs by Hancock et al. (1990), n- and p-MVs possessed similar SDS-PAGE protein-banding patterns that corresponded to those of most major OMPs.

**Encapsulation of exogenous DNA**

To investigate whether MVs could incorporate extracellular DNA, exogenous DNA was used. The preparation of MVs used in this experimentation markedly differed from that used in our previous experimentation that showed plasmid DNA was incorporated from the periplasm; here, p-MVs were first isolated from an overnight culture followed then by DNase treatment. For the exogenous DNA experiments, MVs of PAO1/pAK1900 were isolated from an overnight culture continuously grown in the presence of DNase. By growing cells in the presence of DNase, no extracellular DNA should exist in the supernatant and thus would be accessible to MVs. We do not know whether or not DNase could penetrate the OM or MVs. Interestingly, by PCR analysis (Fig. 5), DNA was not found within MVs isolated by this method. The presence of extracellular chromosomal and plasmid DNA was seen in the ultracentrifuged supernatant of flask A (containing no cells and MVs) (lanes 5, 7). No DNA was present in the cell- and MV-free supernatant of flask B (DNase-treated culture) (lanes 6, 8). Chromosomal and plasmid DNA was associated with MVs isolated from flask A (lanes 9, 11). Again, MVs isolated from the

**Table 2.** Thickness of lipid bilayer, buoyant densities and amount of KDO and NADH oxidase in membrane fractions (OM and PM) and MVs (n-MVs and p-MVs)

<table>
<thead>
<tr>
<th></th>
<th>Thickness of bilayer (nm)*</th>
<th>Buoyant density (g ml⁻¹)</th>
<th>KDO [nmol (mg protein)⁻¹]†</th>
<th>NADH oxidase [nmol min⁻¹ (mg protein)⁻¹]‡‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM</td>
<td>ND</td>
<td>1.170</td>
<td>7.72 ± 0.78</td>
<td>132.50 ± 20.43</td>
</tr>
<tr>
<td>OM</td>
<td>5.39 ± 0.49</td>
<td>1.240, 1.260</td>
<td>190.97 ± 47.15</td>
<td>2.99 ± 2.30</td>
</tr>
<tr>
<td>n-MVs</td>
<td>5.42 ± 0.62</td>
<td>1.240, 1.260</td>
<td>41.02 ± 6.24</td>
<td>0.27 ± 0.32</td>
</tr>
<tr>
<td>p-MVs</td>
<td>5.49 ± 0.52</td>
<td>1.250, 1.265</td>
<td>43.70 ± 6.02</td>
<td>0.07 ± 0.02</td>
</tr>
</tbody>
</table>

ND, Not determined.

*n = 20 (mean ± SD).

†n = 3 (mean ± SD).

‡ One unit = nmol min⁻¹.
DNase-treated culture did not contain internally associated pAK1900 (lane 10). The presence of a product in lane 13 indicated that DNase activity was destroyed before PCR analysis.

To further examine this phenomenon, an additional assay was performed by incubating purified pAK1900 with n-MVs. When DNase treatment was performed, some intact DNA was detected subsequently by PCR (Fig. 6). DNase completely hydrolysed pAK1900 (lane 6), while n-MVs+pAK1900 appeared unaffected by the enzyme (lane 5). These results suggest that plasmid DNA was internalized and protected within the MVs. Fluorometric analysis of these MVs confirmed this finding. n-MVs+pAK1900 contained 1.45 ± 0.71 ng DNA (20 μg protein)^−1, while n-MVs+pAK1900 that were DNase-treated contained 0.57 ± 0.11 ng DNA (20 μg protein)^−1.

n-MVs incubated with pAK1900 contained 1.14 ng more DNA compared with n-MVs alone (Table 1). When both of these MV fractions were DNase-treated, thus only measuring internal DNA, an increase of 0.34 ng DNA in n-MVs+pAK1900 to n-MVs (without pAK1900) was measured (Table 1). These results clearly indicated that exogenous plasmid DNA could be internalized.

It was possible that broken MVs had bound this amount of DNA and, once their membranes had re-annealed, were responsible for encapsulation. For these reasons, the MV stability of p-MVs and OM vesicles was analysed by comparing the number of intact to broken vesicles by cryo-TEM. OM vesicles isolated by isopycnic sucrose centrifugation were used as a standard control for correlation with the p-MVs. By counting over 80 vesicles visualized by cryo-TEM of thin frozen foils, 7.74 % and 7.32 % of p-MVs and OM vesicles were broken, respectively. It is possible that broken MVs were responsible for exogenous DNA encapsulation.

**DISCUSSION**

Our study has shown that during the production of MVs from PAO1/pAK1900, plasmid DNA was compartmentalized within MVs. Previous experimentation has demonstrated that MVs released into the surroundings can transport components such as enzymes and toxins to other bacteria; after attachment and fusion to these cell surfaces, the MV components are delivered into the periplasmic space of recipient cells (Kadurugamuwa & Beveridge, 1997, 1998, 1999). With this in mind, we believed this fusion could also deliver MV-encapsulated DNA into the periplasm, where it would have easier access to the cytoplasm, thereby facilitating genetic transformation. For example, if R-plasmids were contained within MVs, they would be protected from nucleases during transit and their transformation potential should increase, resulting in better lateral transmission of antibiotic-resistance genes to other bacterial species and genera. The possibility that MVs act as a transformation vector would represent a novel mechanism of natural transformation where no chemical or physical manipulation is required. The genetic material would be passed from one bacterium to another via a protected and unbiased system. The OM surrounding the MV would have two functions – a barrier to exogenous nucleases, and a
compatible membrane that can readily fuse to Gram-negative bacteria. Although this system is intuitively logical, our experimentation did not demonstrate DNA transformation via MVs. It seems PAO1 MV-mediated genetic exchange does not occur under our laboratory conditions. Yet, we have used only a limited number of conditions on two types of recipient cells (P. aeruginosa and E. coli) and it is possible that if other incubation conditions or strains were used, transformation could take place.

Our results differ from those of N. gonorrhoeae and E. coli O157:H7, where MVs successfully transformed wild-type strains of their own species. It is possible that PAO1 MVs are inherently different than those of E. coli and N. gonorrhoeae so that they lack transformation capability. It is also possible that the high stringency we used for MV isolation and purity was not achieved by other studies. Using our previous MV isolation procedure (Kadurugamuwa & Beveridge, 1995) an extremely low but consistent level of donor cells was present in the MV samples. It is essential that MV isolation is completely pure and cell-free so that results obtained are due to MVs alone.

Previous studies have indicated that MVs represent novel vaccines for Gram-negative pathogens (Kadurugamuwa & Beveridge, 1999). One concern is that using MVs carrying chromosomal or plasmid DNA would result in the transfer of this information to resident bacteria. Because our experiment shows that P. aeruginosa MVs are transformation inefficient, these MVs could represent an ideal vaccine candidate.

It is interesting to note that DNA is a consistent component of biofilm biomass, and DNA-containing MVs may contribute to this. The matrix that glues the biofilm together is composed of exopolysaccharides, proteins and DNA. Although DNA is not perceived as an essential biofilm component, a study done with alginate-producing P. aeruginosa showed that a consistent proportion of the extracellular material was in fact DNA (Whitchurch et al., 2002). Furthermore, the addition of DNase to established biofilms resulted in the destruction of many early biofilms. Because little cell lysis was observed in these biofilms, a proportion of this DNA may be due to the existence of DNA associated with MVs, which may enhance biofilm production.

To clarify that p-MVs are solely OM-based and contain periplasm without cytoplasmic materials, we showed that the DNA within these vesicles is not a product of cell lysis. If MVs were lysis products, one would expect them to be either double-bilayered, with an OM and PM, or only bound by the PM. Electron microscopy of the MVs indicated the presence of only one bilayer and not a double bilayer. Buoyant density measurements of p-MVs were in a similar range to OM, indicating that the DNA was not contained in PM or PM/OM hybrid MVs. Enzymic measurement of KDO (specific to the OM) and NADH oxidase (specific to the PM) indicated that both n-MVs and p-MVs are OM based and essentially are devoid of any PM. This evidence strongly suggests that MVs do indeed originate from the OM.

Because MVs contain periplasmic components and DNA is...
found in the cytoplasm, its presence in MVs was perplexing. To further clarify the origin of DNA within MVs, two methods of DNA encapsulation were explored: (1) DNA exists in the periplasm and, along with other periplasmic components, becomes encapsulated within an MV, or (2) DNA existing in the extracellular environment is internalized within an MV. It is also possible that a combination of both methods (1) and (2) could occur. Because pAK1900 was not found in p-MVs when cells were grown in the presence of DNase, the model of MV encapsulation of periplasmic DNA was challenged. Also, n-MVs were found to internalize exogenous DNA, possibly by the opening and closing of a small population of vesicles. Recent work done to determine the elasticity of <i>P. aeruginosa</i> MVs found that a small proportion of the MVs (~5%) were collapsed and empty (Stoica et al., 2003). This is consistent with the cryo-TEM findings in this study. It appears that a small amount of DNA is incorporated into MVs by this ‘opening and closing’ phenomenon but the amount of DNA quantified in p-MVs seemed too large for this system to act alone. Most evidence points towards a combination of methods (1) and (2), and we believe the incorporation of DNA within MVs occurs by these two routes.

Clearly, MVs encapsulate DNA and, with further evaluation, the complexity and importance of this internalization will be uncovered.

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**Fig. 5.** PCR analysis of MVs isolated from DNase-treated PAO1/pAK1900 growth culture. Primers used are specific for lac (475 bp; lanes 2, 5, 6, 9, 10, 13) and for oprL (760 bp; lanes 3, 7, 8, 11, 12). Lanes 1 and 14, 1 kb DNA ladder; lane 2, plasmid pAK1900 (control); lane 3, PAO1 genomic DNA (control); lane 4, no template (control); lanes 5 and 7, ultracentrifuged supernatant of flask A (no DNase); lanes 6 and 8, ultracentrifuged supernatant of flask B (with DNase); lanes 9 and 11, MVs isolated from flask A; lanes 10 and 12, MVs isolated from flask B; lane 13, heat-inactivated DNase (control). Molecular sizes in bp are indicated on the left.

**Fig. 6.** PCR amplification of n-MVs following incubation with pAK1900. Primers are specific for lac (475 bp). Lane 1, 1 kb DNA ladder; lane 2, plasmid pAK1900 (control); lane 3, n-MVs alone; lane 4, n-MVs+pAK1900 (16 h incubation); lane 5, n-MVs+pAK1900 (16 h incubation), DNase-treated; lane 6, pAK1900 digested with DNase. Molecular sizes in bp are as indicated on the left.


