DNA-CONTAINING MEMBRANE VESICLES OF *PSEUDOMONAS AERUGINOSA* PAO1 AND THEIR GENETIC TRANSFORMATION POTENTIAL

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ABSTRACT

CHARACTERIZATION OF 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 analyzed for DNA content. PCR analysis using the purified MVs as template indicated that only plasmid DNA is contained within p-MVs. p-MVs could fuse into the outer membrane (OM) of PAO1 and *Escherichia coli* DH5α, delivering the plasmid into the periplasm where it would only have to by-pass the plasma membrane (PM) for effective transformation. However, p-MVs did not transform PAO1 nor DH5α under a variety of transforming conditions.

To ensure that MV-encapsulated pAK1900 was not derived from lysed cells, the physical and ultrastructural differences between both n- and p-MVs were determined. By isopycnic sucrose density gradients, cryo-TEM, characterization of the OM protein and LPS profiles, and enzyme analysis, p-MVs were found to be OM-based. Studies examining the internalization of DNA within MVs suggested that this incorporation could occur by two routes; 1. the MV encapsulates periplasmic DNA and 2. exogenous DNA can be internalized within the MV.

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I dedicate this thesis to my parents and to Nonna Marietta.

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1.0 INTRODUCTION

1.1 General Description of Membrane Vesicles

Membrane vesicles (MVs) are spherical, bilayered sacs that bleb off from the outer membrane (OM) of Gram-negative bacteria. First observed over 30 years ago, these 50 - 250 nm diameter structures, referred to as microvesicles, blebs, OM fragments, membrane vesicles (MVs) or extracellular vesicles, are a common, natural phenomenon of Gram-negative bacteria (Kadurugamuwa and Beveridge 1997; Mayrand and Grenier 1989). They have been observed in laboratory-grown cultures and in natural environments such as freshwater, soil and biofilms (Beveridge 1999; Beveridge et al. 1997). A multitude of genera produce MVs, such as *Pseudomonas* (Kadurugamuwa and Beveridge 1995), *Neisseria* (Devoe and Gilchrist 1973), enterotoxigenic *Escherichia* (Gankema et al. 1980), *Vibrio* (Chatterjee and Das 1967; Kondo et al. 1993), *Borrelia* (Whitmire and Garon 1993), *Haemophilus* (Wispelwey et al. 1989), *Brucella* (Gamazo and Moriyon 1987), *Bacteroides* (Forsberg et al. 1981), *Bordetella* (Morse and Morse 1970) *Shigella, Klebsiella, Serratia, Proteus, Salmonella, Campylobacter, Myxococcus, Aeromonas, Magnetospirillum,* and *Aquaspirillum* (Kadurugamuwa and Beveridge 1997). MVs are composed of outer membrane proteins (OMPs), lipopolysaccharide (LPS), phospholipids and periplasmic material, all components of the producing strain (Fig. 1) (Beveridge 1999; Kadurugamuwa and Beveridge 1995). Since the production of MVs is a common phenomenon and since they are produced by a multitude of Gram-negative bacteria, they should serve a significant function for the bacteria. It would be wasteful to manufacture all the MV structural components and release them from the bacterial surface, all of which are metabolically expensive, for no reason at all. Therefore,

extensive research has attempted to explore and provide evidence of their potential functions. Some possible roles have been suggested such as predation on other bacteria, serum resistance, enhanced virulence effects on tissue during infection and DNA delivery during transformation.

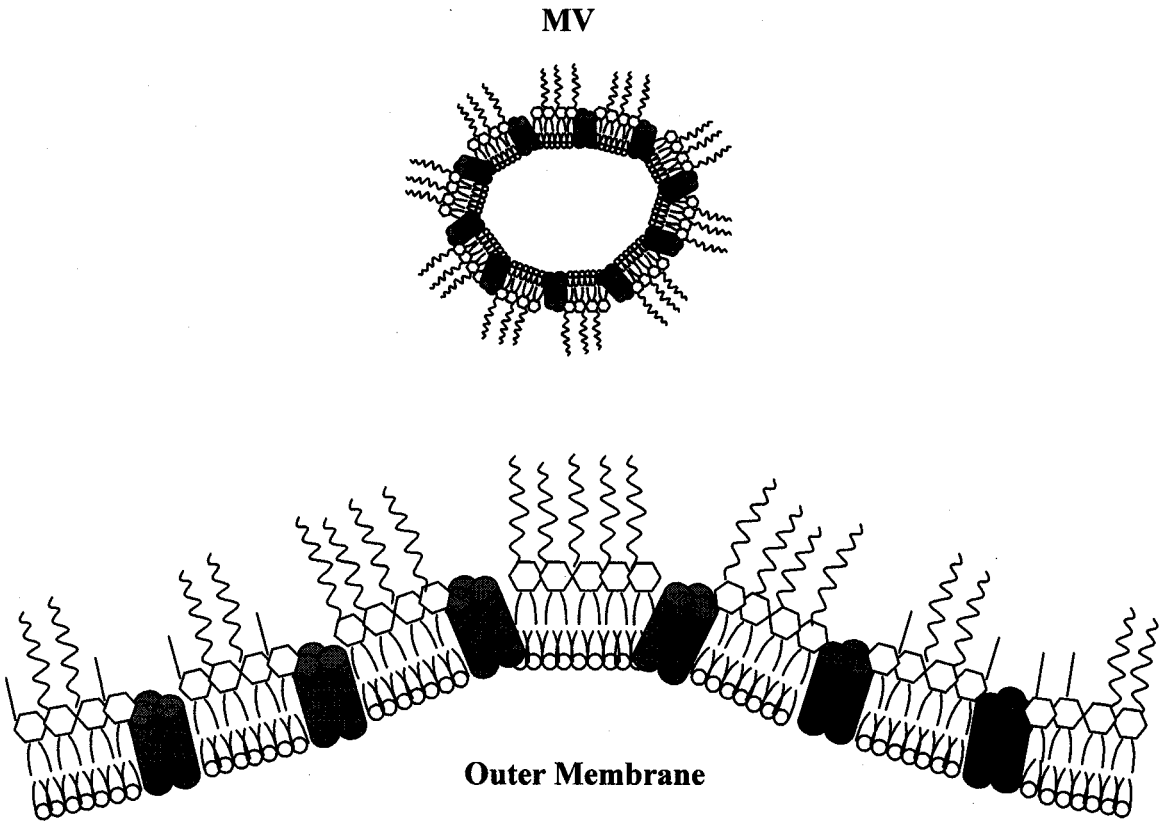

Fig. 1. Structure and composition of the OM of an MV-producing strain (i.e., *Pseudomonas aeruginosa*) and a released MV. Long squiggle lines represent O-side chains of B-band LPS and short straight lines represent O-side chains of A-band LPS. Underneath the LPS are phospholipids and the filled-in cylinders represent porin assemblies.

1.2 Possible functions of MVs

Predation. The possible predatory role of MVs has been investigated. Naturally produced MVs (n-MVs) from various Gram-negative bacteria, which are enriched in peptidoglycan (PG) hydrolase (a cell wall-degrading enzyme), were analyzed for their effects on a variety of Gram-positive and Gram-negative species (Kadurugamuwa and

Beveridge 1998; Li et al. 1998; MacDonald and Beveridge 2002). MVs lysed both types of bacteria, and lysis depended on the PG chemotype of the attacked cells. Chemotypes identical or similar to the chemotype of the MV-producing donor cells were readily lysed (Li et al. 1996). It is possible that PG hydrolase within MVs causes lysis of neighbouring bacteria in order to use these lysis products as a source of organic nutrients essential for growth. Possibly, in natural environments when nutrients required for growth and survival are scarce, a predatory response elicited by MVs could alleviate this stress (Kadurugamuwa and Beveridge 1996).

Serum Resistance. The role of how MVs aid in evasion of host humoral defence systems has also been examined (Petit and Judd 1992b). Serum resistant strains (SRS) of *Neisseria gonorrhoeae* are normally resistant to human serum killing. MVs from SRS demonstrated enhanced binding and removal of anti-gonococcal antibodies IgG and IgM. If these immunoglobulins elicit a bactericidal response, SRS MVs could possibly bind and remove anti-gonococcal antibodies, thereby enhancing the immune evasion potential of this organism (Petit and Judd 1992b).

Virulence. Virulence factors produced by pathogenic bacteria can aid in bacterial colonization and invasion of the host tissue. The synthesis and free diffusion of such macromolecules can be metabolically expensive and wasteful, therefore MVs could provide an alternative route for their delivery.

A wide spectrum of virulence factors have been identified in MVs of *P. aeruginosa*, such as LPS, an endotoxin enriched on the surface of MVs, protease, alkaline phosphatase, phospholipase C (PLC) and pro-elastase found encapsulated within the MVs (Kadurugamuwa and Beveridge 1995; Li et al. 1996). An example of these

components in virulence is the action of PLC and alkaline phosphatase in the liberation of organophosphates from phospholipids (membrane components) (Shortridge et al. 1992; Tan and Worobec 1993). Most of the PLC activity was MV-associated, since the removal of MVs from cell-free supernatants resulted in a 70-80% reduction of enzyme activity (Kadurugamuwa and Beveridge 1995). Furthermore, PLC was found to be hemolytic due to its lytic action on sheep blood. Similarly, alkaline phosphatase activity decreased 50% following removal of MVs from the supernatant, indicating once again that much of this enzyme is associated with MVs. Although protease activity was detected mainly in the MV- and cell-free supernatants, some activity (18%) was found associated with MVs. By immunogold labelling thin sections of MVs with PLC and alkaline phosphatase specific antibodies, the location of these enzymes within MVs was distinguished. The observance of gold particles located at specific areas within the sample indicated that PLC resided within the lumen of the MVs and alkaline phosphatase was associated with the periphery of the MVs. Also, the periplasmic form of elastase (pro-elastase) was also detected within MVs. This provided further evidence that MVs encapsulate periplasmic components, since the active form of this enzyme (elastase) is due to a modification of the enzyme as it crosses the OM (Kessler and Safrin 1988).

Burkholderia cepacia is an opportunistic human pathogen of the lower respiratory tract and is a major cause of lung disease in patients with cystic fibrosis (Gilligan 1991). Like *P. aeruginosa*, *B. cepacia* produces a similar repertoire of virulence factors, including proteases, a lipase, a hemolytic and non-hemolytic PLC, all of which contribute to its pathogenicity (Lonon et al. 1988; McKevitt et al. 1989; Vasil et al. 1990; Weingart and Morris-Hooke 1999). Characterization of MVs isolated from various strains of *B.*

cepacia (C5424, C5632, C5598, 26D7, CEP192, and CEP248) found that many of the above virulence factors were contained within the MVs (Allan 2003). Non-hemolytic PLC and lipase were detected in C5632, C5598, 26D7 and CEP192 MV preparations. When C5632 and CEP192 MVs were removed from cell-free supernatants, a remarkable 88 and 91.2% reduction, respectively, was observed in PLC activity, indicating that most PLC activity is MV-associated. Protease activity was detected in all 6 strains but unlike PLC the majority of the activity remained following removal of MVs, suggesting that the protease was found more commonly in the supernatant as a soluble form than within MVs. The protease was identified as an extracellular zinc metalloprotease which is known to possess a similar function as elastase in *P. aeruginosa*.

Porphyromonas gingivalis (formerly *Bacteriodes gingivalis*), a Gram-negative anaerobe present in the oral cavity, has been linked to periodontal disease (Grenier and Mayrand 1987). MVs produced by this strain exhibit proteolytic activity against collagen. In addition, it was also suggested that MVs increase adherence between sheep erythrocytes and, at the same time, mediate strong attachment between two non-coaggregating cell types of bacteria, *Eubacterium saburreum* and *Capnocytophaga ochracea* (Grenier and Mayrand 1987). Although electron microscopy (EM) showed MVs surrounding these bacteria, this does not necessarily indicate that they act as a bridge between co-existing bacteria. There is some correlation between the presence of MVs and the formation of bacterial cell aggregates but more evidence is needed to clarify the significance of MVs in attachment.

Because an MV is small in structure and contains a concentrated variety of virulence factors it could act as an ideal weapon for a pathogen. By fitting into tiny

crevices of the host tissue and delivering such destructive components it could weaken the tissue and make it more susceptible to infection (Kadurugamuwa and Beveridge 1996).

Toxin secretion and delivery. The efficiency of the secretion and delivery of toxin to host tissues could be enhanced if toxins were concentrated in MVs. Packaging of secreted, active heat-labile enterotoxin (LT) within MVs was demonstrated in enterotoxigenic strains of *Escherichia. coli* (ETEC) (Wai et al. 1995). LT is an AB_5 toxin that binds to G_{M1} ganglioside of the gut epithelial cells via the B_5 subunits (Horstman and Kuehn 2000). The enzymatic activity of the A subunit creates an increase in cyclic AMP levels which causes an efflux of water and electrolytes, resulting in diarrhea. Although the structure of this toxin is well understood, little is known about the mode of LT secretion. LT gathers in the bacterial periplasm but is not excreted from the bacteria in a soluble form (Horstman and Kuehn 2000). Analysis of the culture supernatant identified that 95% of the LT activity was associated with MVs, which suggests that MVs act as enterotoxin carriers (Horstman and Kuehn 2002). Furthermore, LT was also found on the cell surface, bound to LPS. Because ETEC MVs are similar in composition to the outer surface of ETEC cells, they also contain LT tethered by LPS. Potentially, an MV-LT association with G_{MI} could lead to an interaction with a host cell (Horstman and Kuehn 2002). The formation of an LT-induced bridge could cause the host cell to internalize the MV, thereby intoxicating the cell.

Similar observations of toxin-concentrated MVs were also observed in *Helicobacter pylori* and *Actinobacillus actinomycetemcomitans*. Biologically active VacA, a cytotoxin which can insert itself into lipid bilayers, induce permeability of

epithelial cells (Papini et al. 1998) and also cause vacuolation of host cells (Molinari et al. 1997), is contained within *H. pylori* MVs (Keenan et al. 2000a). By SDS-PAGE analysis the presence of porin proteins in MVs resulted in speculation that MVs could transport and potentially release these proinflammatory and cytokine-inducing proteins to target cells. Because porins are proteins that form channels across the OM, if these molecules are small enough (i.e., VacA), they could travel through and out of these channels to target cells. Leukotoxin is expressed on the OM of *A. actinomycetemcomitans* and it is highly toxic to lymphocytic and monomyelocytic cells. MVs produced by this strain also possess active leukotoxin. The addition of these MVs to HL60 cells demonstrated toxicity levels 4-5 fold higher than the OM (Kato et al. 2002; Nowotny et al. 1982). This observation could be due to surface-associated toxin as well as internalized leukotoxin within MVs. This combination would result in enhanced toxic activity as compared to that associated with the OM of *A. actinomycetemcomitans*. Previous studies have demonstrated the binding of leukotoxin to β2-integrin, a cell surface receptor on human immune cells (Lally et al. 1997). Potentially, the leukotoxin/integrin interaction could allow the attachment of MVs to the target cell surface, initiating a leukotoxic cascade, and possibly contribute in the delivery of additional virulence factors to the cell (Kato et al. 2002). Unpublished work by Sato et al. indicate that the incubation of *A. actinomycetemcomitans* MVs containing fluorescently labelled phospholipid with HL60 cells resulted in the rapid incorporation of this fluorescent lipid into the cell membranes. This fusion could be due to the initial association of the leukotoxin with its receptor. Further studies are needed to examine

whether the luminal components of these MVs are also delivered into the HL60 cell cytoplasm.

DNA delivery. Another possible MV function is their involvement in the distribution of DNA to other bacteria. DNA has been found within MVs isolated from competent (*N. gonorrhoeae* and *Haemophilus influenzae* and *Haemophilus parainfluenzae*) and noncompetent (*P. aeruginosa* and *E. coli* O157:H7) bacteria (Dorward et al. 1989; Kadurugamuwa and Beveridge 1995; Kahn et al. 1983; Kolling and Matthews 1999; Lo et al. 1998). These observations sparked the idea that MVs play a role in DNA delivery, which could result in genetic transformation of the recipient cell. MVs acting as transforming agents would combat the need for competence due to the similar composition of the MV and recipient cell surfaces (i.e., OM). Furthermore, the DNA contained within the MV would be protected from digestion by exonucleases. A detailed assessment of the previous research, which examines the function of MVs as DNA delivery and transformation agents, will be discussed in depth in Chapter 3.

As can be seen from the previous discussion, MVs have demonstrated the ability to package a variety of lytic and destructive components such as enzymes and toxins as well as demonstrating the ability to evade host defenses and contain DNA. Thus, MVs may contribute tremendously to bacterial pathogenicity, toxicity and survival.

1. 3 Possible medical applications of MVs

Recognizing the structure and natural functions of MVs, it should be possible to design MVs for various beneficial applications, possibly as therapeutics and preventatives. The addition of gentamicin at inhibitory levels (4X the MIC) to a growing culture of *P. aeruginosa* resulted in the presence of this aminoglycoside antibiotic within

MVs (g-MVs). These MVs contained the antibiotic as well as potent autolysin. The packaging of gentamicin, which targets the ribosome and thus interferes with protein synthesis and an autolysin which hydrolyzes the PG, represents an ideal therapeutic vehicle where MVs could deliver these destructive agents to both Gram-positive and Gram-negative pathogens. As indicated above the use of such MVs did result in killing of both types of bacteria (Kadurugamuwa and Beveridge 1996). Another advantage to this system is that MVs act as a protective barrier for entrapped gentamicin and the autolysin from any degradative extracellular enzymes. With these reasons, MVs represent a novel therapy where a concentrated amount of antibiotic and other potent components are directly delivered to persistent pathogens (Beveridge 1999, Kadurugamuwa and Beveridge 1996). One major concern with the use of MVs as an antimicrobial therapy is that LPS, a major component of MVs is endotoxic. Administering LPS-containing MVs to a host could cause septic shock, resulting in systemic inflammation and multi-organ failure (Horn et al. 1996). However, if MVs can be delivered by an oral route, the potential endotoxicity of the MVs could be reduced or eliminated (Beveridge 1999).

The treatment of infections caused by intracellular pathogens is another area in which g-MVs could be of some benefit. Since many antibiotics are unable to penetrate mammalian cells (where intracellular pathogens persist), the entrapment of antibiotics within MVs could circumvent the poor penetration of such drugs (Kadurugamuwa and Beveridge 1998). Similar to *P. aeruginosa*, the addition of gentamicin to a culture of growing *Shigella flexneri* resulted in the formation of g-MVs (Kadurugamuwa and Beveridge 1998). The ability of g-MVs to deliver this gentamicin into infected

mammalian cells was investigated. *S. flexneri* g-MVs were added following infection of the human epithelial cell line, Henle 407, with the intracellular pathogen *S. flexneri.* As viewed by EM of immunogold labeled antibodies specific for LPS, these g-MVs acted exactly like intact *S. flexneri* cells; they attached to tissue culture cells, formed an invagination and were taken up into a phagosome. Immunoblot analysis using antibodies specific for the bacterial invasion proteins IpaB, IpaC, and IpaD indicated these proteins are g-MV components. Ipa proteins are OMPs of *S. flexneri* and are essential for the pathogen to penetrate epithelial cells and escape phagocytic vacuoles (Watarai et al. 1995). After uptake, g-MVs were present in the vacuoles, attached to the vacuolar membrane and, eventually, reached the cytoplasm of the target cells. Here, growth of *S. flexneri* was inhibited, which suggests that the delivered gentamicin acted on the pathogen. Therefore, the ability of g-MVs to attach, penetrate and attack intracellular pathogens represents a novel system of antibiotic delivery that can combat persistent intracellular infections.

The discovery that MVs fused with live, attenuated strains of bacteria sparked the idea that MVs could administer an efficient and effective vaccine delivery system (Kadurugamuwa and Beveridge 1999). MVs isolated from *S. flexneri* and *P. aeruginosa* were found to fuse with *Salmonella typhi* Ty21a, *Salmonella enterica* serovar Typhimurium *aroA* and *E. coli* DH5α. Fusion of the MVs with these vaccine strains resulted in the integration of foreign antigens from the two MV types into the surface of the recipient strains. An immune response to these foreign antigens was also investigated when MVs of PAO1 and *E. coli* and whole cells of *S. enterica* were subcutaneously injected into BALB/c mice (Fu et al. 2000). Strong immunogenicity and antigenicity

responses against surface cell antigens (primarily the LPS) were detected by ELISA, ELISPOT of IL-4 and IFN-γ, and a proliferation assay, suggesting that these antigens represent a good vaccine candidate. In addition, the endotoxicity of MVs was measured via the *Limulus* amoebocyte assay and rabbit skin tests and showed that MVs are much less endotoxic than isolated LPS. While these results are encouraging, more *in vitro* and *in vivo* endotoxicity tests need to be performed to ensure the safety of this treatment. Furthermore, more research is required to investigate whether oral delivery of these vaccine strains may elicit a desired mucosal immune response against both the vaccine strain and the MV parent strains (Beveridge 1999; Kadurugamuwa and Beveridge 1999).

With the increasing emergence of antibiotic-resistant strains and the need for safer and more protective vaccines, research involving MVs for the management of a variety of problematic pathogens has recently emerged. MVs from *Bordetella pertussis*, *Helicobacter felis*, *H. pylori* and *Neisseria meningiditis*, all of which represent a significant threat to the health of humans and animals, have been analyzed for their ability to illicit an immune response (Hozbor et al. 1999; Keenan et al. 1995; 1998; 2000a, 2000b; Mirlashari et al. 2001). For a more detailed analysis of this area of work please refer to the references provided.

1.4 *P. aeruginosa* PAO1 MVs in more detail

P. aeruginosa is a ubiquitous, Gram-negative, opportunistic pathogen, which persists in cystic fibrosis, trauma and burn patients. During growth, *P. aeruginosa* produces and releases MVs which are liberated from the OM into the external environment (Beveridge 1999; Kadurugamuwa and Beveridge 1995). Extensive research on MVs from *P. aeruginosa* has made it the model organism for examining the

characteristics and possible functions of MVs. As indicated above, *P. aeruginosa* MVs package periplasmic components such as alkaline phosphatase, a 26 kDa autolysin (PG hydrolase), protease, PLC, pro-elastase, and DNA (Kadurugamuwa and Beveridge 1995; Li et al. 1996). These components are contained within an OM enclosed structure which measures approximately 50 - 250 nm in diameter (Beveridge and Kadurugamuwa 1996). Although *P. aeruginosa* PAO1 possesses two chemically and immunologically distinct types of LPS, a short chained and neutral charged A-band as well as a longer chained electronegative B-band (Lam et al. 1992, Rochetta et al. 1999), only B-band LPS is associated with the outer surface of n-MVs (Kadurugamuwa and Beveridge 1995). A hypothetical model (Fig. 2) designed by Kadurugamuwa and Beveridge (1995) describes the formation of PAO1 MVs.

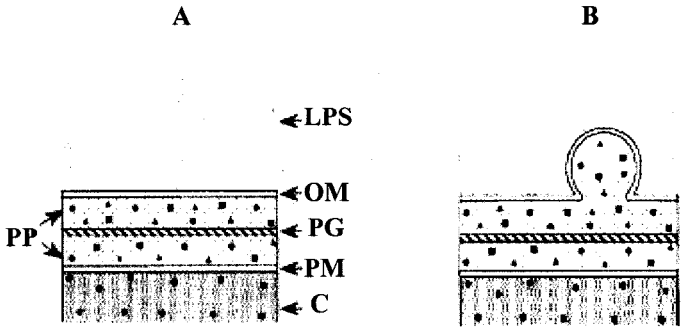

Fig. 2. Model of n-MV formation. (A) The cell wall before MV formation and (B) an n-MV blebbing from the OM, which entraps periplasmic material. C, cytoplasm; PM, plasma membrane; PG, peptidoglycan; OM, outer membrane; LPS, lipopolysaccharide; PP, periplasm; PLC, •; protease, ■; alkaline phosphatase, ▲. (Reprinted from Beveridge and Kadurugamuwa 1995).

The O-side chains of B-band LPS are composed of a trisaccharide repeat of two uronic acid residues and one N-acetyl-fucosamine giving each trimer an overall single negative charge (Knirel and Kochetkow 1994, Rochetta et al. 1999). These O-side chains

vary in length and may extend up to ~ 40 nm from the cell surface (Lam et al. 1992). The core and lipid A regions of B-band LPS contain exposed phosphoryl and carboxyl groups (Karunaratne et al. 1992, Sadovskaya et al. 1998). The net result is that B-band LPS is highly electronegative (Kadurugamuwa et al. 1993). In order to stabilize B-band LPS, Mg^{2+} and Ca^{2+} neutralize these anionic sites by salt-bridging adjacent LPS groups. However, because B-band LPS is highly electronegative and highly variable in length, complete salt-bridging of all adjacent side chains is virtually impossible (Kadurugamuwa and Beveridge 1995). This results in repulsion of the upper polar portions of the LPS molecules, pushing the upper region of the molecule apart and forcing the underlying hydrophobic regions closer together. Therefore, small areas of the OM that contain localized B-band LPS are forced into high-curvature structures, causing these regions to bleb (Kadurugamuwa and Beveridge 1996). As the MV forms, periplasmic material becomes compartmentalized within the structure and is then liberated into the external environment.

The quantity of MVs secreted by PAO1 can be elevated to 3-5-fold more than natural yields with the addition of gentamicin (Martin and Beveridge 1986; Kadurugamuwa and Beveridge 1995). Gentamicin is a cationic molecule that competes with the Mg^{2+} and Ca^{2+} ions that cross-bridge adjacent LPS molecules on the bacterial OM (Kadurugamuwa et al. 1993). Binding of gentamicin to these regions causes a destabilization of the membrane that results in perturbation and blebbing (Kadurugamuwa et al. 1993). Like n-MVs, g-MVs also contain periplasmic enzymes and B-band LPS. However, g-MVs also incorporate some A-band LPS and a small quantity of gentamicin as they form (Kadurugamuwa and Beveridge 1995). Both n-MVs and g-

MVs of PAO1 displayed a bacteriolytic effect on Gram-negative, Gram-positive and S-layered Gram-positive strains due to the 26 kDa PG hydrolase (Kadurugamuwa and Beveridge 1995; Kadurugamuwa et al. 1998). As mentioned before, lytic potency of MVs depends on the host bacterium's PG chemotype. MVs produced by PAO1 do not kill other PAO1 cells, as this enzyme should be recognized by the cell as one of its own normal autolysins and would be regulated. The exact mechanism of autolysin regulation has not yet been determined. However, the ability of PAO1 to regulate the activity of this 26 kDa autolysin has been demonstrated by Western immunoblots and PG zymograms of synchronously growing cells (Li et al. 1996). Although this enzyme is present throughout the cell cycle, it is only active during cell division (Li et al. 1996).

The attachment of MVs and the release of MV components to Gram-positive and Gram-negative bacteria are proposed to be different (Fig. 3). When the anionic B-band LPS-enriched surface of an MV encounters Gram-negative and Gram-positive bacteria, the MVs should readily adhere to these bacterial cell surfaces, which are both rich in Mg^{2+} and Ca^{2+} ions (Beveridge 1981; Beveridge 1988; Ferris and Beveridge 1986). Gram-positive bacterial cell walls contain an abundance of these ions associated with PG and teichoic and teichuronic acids. Therefore, contact with such cell walls should result in immediate adherence and the formation of salt-bridges via Mg^{2+} and Ca^{2+} ions. By forming more inter-LPS salt bridges, destabilization of the high-curvature MV would result, forcing it to break open and release its autolysin to degrade the PG beneath this adherence site (Kadurugamuwa and Beveridge 1996). On the other hand, when MVs attach to Gram-negative bacteria, fusion is favoured because the recipient's OM is compatible with the MV bilayer. The luminal contents are, thus, liberated into the

periplasm, allowing PG hydrolase to diffuse to and hydrolyze the PG layer at a number of different sites (Kadurugamuwa and Beveridge 1996). As long as the recipient bacteria are actively growing, the lytic action of n-MVs is minimal since the PG hydrolase is diluted out by cell wall turnover as cell elongation ensues.

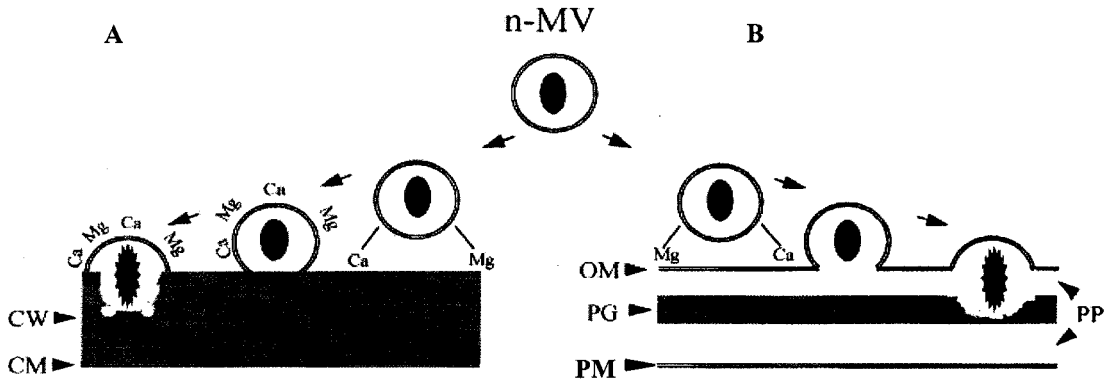

Fig. 3. Model explaining the interaction of n-MVs with (A) Gram-positive and (B) Gram-negative surfaces. PM, plasma membrane; CW, cell wall; OM, outer membrane; PG, peptidoglycan; PP, periplasm; Ca, calcium ion; Mg, magnesium ion. (Reprinted from Kadurugamuwa and Beveridge 1996).

A recent study attempted to further evaluate the interaction of MVs with Gram-positive bacteria. The bactericidal effect of PAO1 g-MVs against four Gram-positive organisms was investigated. Bactericidal assays revealed that *Bacillus subtilis* and *Staphylococcus aureus* were vulnerable to g-MV mediated killing, *Listeria monocytogenes* was slightly vulnerable, whereas *Enterococcus hirae* was completely unsusceptible to g-MVs as well as soluble gentamicin (MacDonald and Beveridge 2002). In the first three cases, g-MVs were generally more potent than soluble gentamicin treatments. EM of thin sections demonstrated the attachment of MVs to all four strains but only *B. subtilis* and *S. aureus* showed cell wall degradation and lysis. This work concluded that the interaction between Gram-positive organisms and g-MVs differed and

was dependent on cell surface characteristics, such as hydrophobicity, the mode of bacterial growth and the ability of the packaged autolysin to degrade the target PG (MacDonald and Beveridge 2002). In addition, although g-MVs were not effective at killing all organisms studied, this system does represent a promising therapeutic alternative for specific Gram-positive bacteria.

There may also be a beneficial effect on the transfer of substances packaged within MVs to recipient cells. An example of this is the identification of β-lactamase packaged in *P. aeruginosa* MVs that was transferred to non-β-lactamase producing strains (Ciofu et al. 2000). β-lactamase is a periplasmic enzyme which inactivates β-lactam antibiotics before they reach the penicillin-binding proteins on the plasma membrane (PM) (Ciofu et al. 2000). MVs containing β-lactamase could then be transferred to neighbouring non-producing β-lactamase Gram-negatives where they could then withstand antibiotic action (Beveridge 1999).

1.5 DNA in PAO1 MVs

The presence of genetic material within MVs isolated from *P. aeruginosa* was first discovered by Kadurugamuwa and Beveridge (1995). Comparison between g-MVs and n-MVs found that DNA was contained in both MV types, but g-MVs contained slightly more (strain ATCC 19660; 16.12 ±1.02 ng DNA/mg protein) than n-MVs (14.35 ± 0.49 ng DNA/mg protein). The encapsulation of DNA within MVs was a surprising observation, considering that DNA resides in the cytoplasm and that MVs are primarily composed of OMPs and periplasmic material.

Based on this initial study, Lo et al. (1998) investigated whether MVs produced by *P. aeruginosa* PAO1 carrying plasmid pCC75 contained this plasmid DNA. pCC75

contains *algA* which encodes for an enzyme in the alginate biosynthetic pathway (Chitnis and Ohman 1993). Specific primers were designed for *algA* and PCR analysis on MVs produced from PAO1 and PAO1/pCC75 was performed. An amplification product was observed with PAO1/pCC75 MVs even when the MVs were DNase treated. A brighter product was obtained when exogenous pCC75 was added to the reaction, which implied that MVs may contain endogenous DNA not susceptible to external DNase treatment. This DNA could be plasmid or chromosomal in origin because *algA* is found on both pCC75 and the PAO1 genome. MVs from PAO1 also produced a PCR product but only when there was no DNase treatment or when exogenous plasmid DNA was added. Because DNase treated MVs from PAO1 did not produce any PCR product, it suggested that there was no chromosomal or plasmid DNA contained within these MVs. The PCR product which was obtained with untreated (no DNase) PAO1 MVs could be due to the presence of small amounts of chromosomal and/or plasmid DNA naturally associated with the external surface of MVs (Lo et al. 1998). The association of DNA with these MVs requires further study to clarify how DNA associates with *P. aeruginosa*.

1.6 DNA associated with MVs of other bacterial species

Before the study of DNA-containing MVs from PAO1, the presence of DNA within MVs from other bacterial systems such as *N. gonorrhoeae*, *H. influenzae* and *H. parainfluenzae* was observed (Dorward et al. 1989; Kahn et al. 1982; 1983). Association of DNA with MVs from *E. coli* O157:H7 has been recently been found (Kolling and Matthews 1999; Yaron et al. 2000).

N. gonorrhoeae is a naturally competent bacterial pathogen that causes the sexually transmitted mucosal and disseminated infections of gonorrhea. Like many other

Gram-negative bacteria, it produces MVs that contain LPS, other surface antigens and DNA. When applied to a sucrose gradient and centrifuged, isolated MVs were separated into two fractions, BI and BII (buoyant densities of 1.12 g/ml and 1.30 g/ml, respectively) (Dorward et al. 1989). By EM, the BI MVs appeared flattened and lobed with dimensions averaging 26 by 62 nm, while the BII fractions appeared spherical (78 nm in diameter) and elongated or tubular (30 by 196 nm in diameter). Comparison of their protein profiles indicated that these two fractions were different in composition. BI MVs did not contain major OM components such as PI porin, PII OMP and LPS, but did contain a 60 kDa protein presumed to be cytoplasmic. This suggests that BI MVs may originate from the PM unlike BII MVs, which have an OM origin (Dorward et al. 1989). When analyzed, BI and BII MVs contained linear and chromosomal DNA, including 4.2- and 7.1 kb plasmids along with small amounts of RNA (Dorward and Garon 1989).

In order to determine whether the isolated DNA was contained within the MVs and not merely externally associated with the enclosing membrane, exogenous DNase treatment and EM were performed. The former experiment demonstrated complete hydrolysis of DNA associated with the BI MVs, while plasmids from BII were unaffected by this treatment (Dorward et al. 1989). The Kleinschmidt technique was used on samples of BI and BII for EM. This technique is an effective way of untangling nucleic acids and spreading them on EM grids (Bradley 1965). Here, the sample is spread by surface tension and the nucleic acid strands present are distributed evenly over the grid as linear strands or intact coiled circles. The grid is then rotary shadowed with platinum or tungsten which clearly delineates the macromolecules for EM (Bradley 1965). This method allowed visualization of supercoiled, open circular and linear molecules in both

BI and BII fractions. When BI and BII were cross-linked by glutaraldehyde, a treatment which prevents the release of membrane-bounded internal DNA, DNA was visualized in BI samples but not in BII samples. These results indicated that DNA is externally associated with BI MVs whereas DNA is found within the BII MVs (Dorward et al. 1989).

H. influenzae and *H. parainfluenzae*, both involved in respiratory tract infections, are also known competent bacteria. *Haemophilus* prefers to bind to homospecific DNA that contains an 11-bp uptake sequence. Specific DNA receptors located on the cell surface are responsible for the uptake of this DNA (Danner et al. 1980). When membranous extensions were found forming on these bacterial surfaces it was suggested that these structures could play a role in the binding and uptake of exogenous DNA. Initially when cell competence in *H. influenzae* was induced (transfer of exponential-phase cultures into a synthetic medium (MIV) that does not contain several ingredients necessary for sustained growth) the normal smooth surface of the cells became wrinkled and MVs developed on the cell surface (Deich and Hoyer 1982). Following the return of the cells to normal growth medium (no longer favouring competence), these extensions were liberated from the OM as vesicles, and the smooth nature of the surface returned. When thin sections of competent and noncompetent cells were examined by EM, a greater number of MVs (approximately 5 times more) were found on the surface of competent as compared to noncompetent cells (Kahn et al. 1982). An average of 10-12 of these extensions appeared about 35 nm from the cell surface with an approximate diameter of 20 nm. MVs were seen as bilayered structures located above points of fusion between the PM and OM. At this junction, a pore structure with an opening of 5 nm was

also observed (Kahn et al. 1982). The formation of these membranous structures that remained attached to the surface of competent cells was proposed as a method to capture and protect donor DNA. In fact, when donor DNA was added to competent *H. parainfluenzae* cells, the surface MVs disappeared. To investigate this, [^{32}P]-labelled plasmid DNA was added to competent cells and the fate of the DNA was monitored by autoradiography (Kahn et al. 1983). Within the first 3 minutes the majority of the donor DNA attached to and entered the MVs. Following the internalization of DNA, the DNA remained in a protected state where it was resistant to external DNase, high salt and cellular restriction enzymes. The treatment of cells with organic solvents (phenol/acetone) followed by buoyant density centrifugation resulted in the isolation of a pellet which contained [^{32}P]-DNA associated with a homogenous mixture of 100 nm-sized MVs (Kahn et al. 1983). The discovery that DNA enters the cell via these MVs and could be recovered from disrupted cells in a protected state sparked the idea that these structures could be involved in genetic transformation and, hence, were re-named transformasomes. The proposed mechanism of transformasomes as DNA-transforming agents will be discussed further in Chapter 3.

Vesicle shedding *Haemophilus* mutants were studied by Kahn et al. (1979) and Concino and Goodgal (1982). Competence deficient mutants of *H. influenzae* and *H. parainfluenzae*, *com*-51 and *com*-10, respectively, produced vesicles that were no longer attached to the producing cell. These mutants were derived from nitrosoguanidine mutagenesis and were characterized as competence deficient mutants due to their inability to take up DNA after competence developing procedures. DNA binding was tested by incubation of [^{3}H]-labelled *H. influenzae* DNA with the MVs which were then

filtered through nitrocellulose filters, the filters dried and counted for radioactivity (Kahn et al. 1979). The amount of donor DNA retained by both *com*-10 and *com*-51 transformasomes was similar to that reported for wild-type competent. Although *com*-51 cells were unable to take up *H. influenzae* DNA after being subjected to competence developing procedures, these cells produced transformasomes capable of binding DNA (Concino and Goodgal 1982). Analysis of the protein profiles of *com*-51 cells and transformasomes indicated that a 29-kDa competence specific protein was present in the transformasome preparation but not in the cell membrane extract of *com*-51 cells. This protein was also seen in the wild-type cells and has been implicated as a DNA binding receptor.

The mechanism by which DNA is packaged into these structures is still unknown. It is clear that the production of transformasomes in *Haemophilus* represents a complex system where the state of competence seems a requirement in the formation of transformasomes, the internalization of DNA in a protected state, and the possible incorporation of this genetic material into the genome.

Recently, the research of Kolling and Matthews (1999) and Yaron et al. (2000) focused on MVs from a naturally noncompetent bacterium, *E. coli* O157:H7. This Gram-negative pathogen causes diarrhea, vomiting, and abdominal cramping and can lead to a more serious complication, hemolytic-uremic syndrome (Su and Brandt 1995). By EM, MVs from O157:H7 were observed as spherical structures with darkly stained centers, ranging in size from 20 to 100 nm. DNA was detected within these vesicles using a fluorometric assay. Because the DNA was detected only in intact DNase-treated vesicles, thus protected from exogenous DNase, DNA was believed to be contained within the

vesicles. Additional information relating to the origin of the MV DNA was investigated using PCR. Primers based on the nucleotide sequence of *eae*, *stx1*, *stx2*, *uidA*, *hylCA*, *mobA*, L7095 were designed and PCR amplification was then performed on *E. coli* O157:H7 MVs. The amplification of correctly-sized PCR products for these genes verified their presence within MVs.

These observations are perplexing since the genes are of chromosomal, bacteriophage and plasmid origin. *eae* (encoding intimin) and *uidA* (encoding β-D-glucuronidase) are chromosomal genes, *stx1* and *stx2* (both encoding Shiga toxin) are bacteriophage associated genes, and *hlyCA* (encoding pore forming cytolysin), *mobA* (encoding a mobility protein), and L7095 (encoding a protein with a cytotoxin active site) are located on plasmids. Therefore, there does not seem to be a cellular-directed mechanism for incorporation of a specific type of DNA; DNA entrapment is likely random.

Although little research has been performed in the area of MV characterization in *N. gonorrhoeae*, *H. influenzae*, *H. parainfluenzae* and *E. coli*, these studies combine to strongly indicate that the association of DNA within these membranous structures is a common phenomenon. These initial studies reinforce the need for further experimentation to uncover the overall importance of vesicles in DNA transfer.

1.7 Rationale for present study

Previous experimentation has demonstrated that MVs released into their surroundings can transport components such as enzymes and toxins to other bacteria by MV attachment and fusion to other cell surfaces (Kadurugamuwa and Beveridge 1998; 1999). With this in mind, it is possible that MVs can deliver small quantities of DNA to

other bacteria, protecting it en route because of the surrounding OM. Fusion would liberate DNA into the periplasm of a recipient Gram-negative bacterium allowing it to eventually enter the cytoplasm thereby conferring a change in the recipient's cell genome. For plasmids, as the smallest and most readily mobile DNA elements, the possibility that MVs act as a delivery vehicle would represent a novel mechanism of natural transformation where no chemical or physical manipulation is required to induce cell competency. Because naturally competent bacteria are genetically programmed to permit the efficient uptake of macromolecular DNA (Dubnau 1999) and noncompetent bacteria lack this equipment, MVs may provide a solution to fulfil this requirement. Accordingly, these vesicles would allow bacteria an ingenious method to pass along their genetic material using a protected and unbiased system. The OM layer surrounding the MV would provide two functions – not only would it provide a barrier to any exogenous nucleases, but it would also act as a compatible membrane that can easily fuse to Gram-negative bacteria.

Since the initial discovery of DNA association with MVs isolated from *P. aeruginosa*, little work has been done to further characterize this system and to determine its transformation potential. Therefore, the objectives of the present study were to investigate MVs from PAO1/pAK1900 (p-MVs) and their DNA content and also to determine if these p-MVs transform PAO1 and *E. coli* DH5α (as recipient bacteria). In addition, this work attempted to determine physical and compositional differences (such as buoyant density, membrane enzymes, DNA content and ultrastructure) between p-MVs and n-MVs. Because it is unclear how cellular DNA, a component which resides in the cytoplasm is incorporated within these periplasm-containing MVs, the origin of p-

MVs was evaluated.

Because an MV isolation procedure must be designed to ensure no cellular contamination from the parent MV-producing cells, the previous protocol used to isolate MVs (Kadurugamuwa and Beveridge 1995) required more stringent precautions. Subsequently, this work was to develop a system to isolate MVs that is completely free of donor cells to ensure that transformants obtained following p-MV fusion experiments were due to plasmid transfer via MVs and not due to a contaminating source.

2.0 A CELL-FREE MV ISOLATION SYSTEM AND DETERMINATION THAT INTERNALIZED DNA WITHIN PAO1/pAK1900 MVS IS PLASMID BASED

2.1 Background

Only a handful of studies have looked at the incorporation of DNA within an MV and whether this DNA-vesicle structure has the potential to transfer DNA to other bacteria. As mentioned in the introductory chapter, researchers working with MVs from *N. gonorrhoeae* and *E. coli* report that MVs contain DNA and RNA giving them the ability to exchange the genetic material contained within them to other bacterial species and genera.

One major concern which must be addressed before clearly stating that these observations represent the true nature of MV-DNA is whether isolated MV preparations are contaminated with donor cells. Any contamination of the donor strain (no matter how small) in the MV preparation would result in false positive results in experiments done to identify and quantify DNA assumed to be associated with MVs. As well, transformation studies using these MVs that contain the parent strain could also contribute to false transformants or be a factor in transformation. Our laboratory is one of the few that follows each step of MV isolation and we have a true appreciation of how easy it is to contaminate an MV preparation with an extremely low number of donor cells.

The first work done in this area focused on the MVs of *N. gonorrhoeae* (Dorward et al. 1989). MVs were isolated by sucrose density gradient centrifugation, a method which may result in samples containing cells. First, colonies were picked off plates, resuspended in buffer, vortexed and centrifuged twice to remove whole cells. No filtration of the supernatant was performed to ensure removal of whole cells; therefore,

when this supernatant was layered onto sucrose gradients, it is likely that many cells still remained in solution. Following sucrose gradient centrifugation, the two bands containing MVs were removed. The BII MVs, found at a density of 1.30 g/ml located near the bottom of the tube, would be close in proximity to any cells that had settled to the bottom of the tube. The protocol does not mention how the samples were kept separate during removal. If they were removed from dripping the sample from the bottom, BI and BII MV fractions containing whole cells is likely. If the bands were removed from the top by syringe, there was also a possibility that while removing the BII fractions, some whole cells resting at the bottom of the tube would also be suctioned. Even though no cells were observed by EM, which is a low sampling technique, Dorward et al. (1989) indicated that the overwhelming pleomorphism in each MV fraction made microscopic analysis of fraction purity difficult, especially with BII MVs where pleomorphism was most extensive. These isolated MVs were directly DNase treated, DNA was extracted with phenol/chloroform and samples were observed by electrophoresis on ethidium bromide stained gels. If the fraction purity was at all compromised (containing *Neisseria* cells), then whole cell DNA and MV-DNA would both be represented in the extract, thereby resulting in erroneous results. On the other hand, in plasmid transfer experiments, MVs were isolated differently. MVs were prepared by filtering broth culture with 0.22 μm porosity filters eliminating the possibility of whole cells contaminating the sample. A discussion of these transformation results will be discussed further in Chapter 3.

The first study of MVs from *E. coli* O157:H7 by Kolling and Matthews (1999) reported that MVs from this strain contain chromosomal, plasmid and bacteriophage associated DNA and transfer of this MV-DNA to wild-type *E. coli* lacking these genes. There are some concerns with this work. First, they followed the MV isolation protocol developed by Kadurugamuwa and Beveridge (1995). An overnight culture of exponentially growing cells is centrifuged to remove whole cells, the supernatant is filtered to remove any remaining cells and the resulting filtrate is spun in an ultracentrifuge. Following ultracentrifugation, MVs are concentrated as a small pellet. Although this method does allow for the isolation of MVs, in our present work, it has been observed that a few but consistent number of parent cells accompany the MV preparation. Therefore, although results indicate that *E. coli* O157:H7 MVs encapsulate DNA and possess the ability to transform other *E. coli,* this may not represent the actual MV-DNA system.

Here are some of my concerns with Kolling and Matthews' research. Isolated vesicles were checked for the presence of *E. coli* by surface plating of the vesicle suspension on TSA plates and also by viewing the samples by EM. Surprisingly, by these methods, no contaminating cells were observed. Considering that the MV isolation protocol was followed as outlined by our laboratory, it is difficult to comprehend how no cells were present. Assuming that the parent strain is present in the MV preparation, though in minute quantities, this could result in a misrepresentation of the actual DNA within an MV sample. An example of this is the authors' quantification of DNA by the PicoGreen assay in which surface associated (MV untreated and MVs DNase treated) and encapsulated (MVs DNase treated and lysed with guanidium thiocyanate regent (GES))

DNA was determined. DNA was detected only in the intact and lysed (pre-DNase treatment) MVs. These findings in combination with the absence of DNA found in DNase-treated and lysed MVs suggested that DNA was compartmentalized within MVs. Now, if the parent strain was also present in this MV sample, the use of 5 M GES to lyse MVs would also release DNA from whole cells (Pitcher et al. 1989). The DNA released by this method would result in quantification of DNA that is not only MV-associated but also cell-associated.

Another concern of this work is the method by which the DNase activity of MVs was stopped. Following the incubation period of DNase with MVs, EDTA was added to the reaction at a final concentration of 0.08 M. Leive (1965a) observed that when *E. coli* was incubated with only 0.005 M EDTA for 2 min, a drastic effect on cell integrity resulted. EDTA acts as a chelator and it displaces metal cations that participate in non-covalent bonds which hold together various layers of the Gram-negative cell wall (Eagon 1969). The interaction of EDTA results in the rapid and extensive change in composition of the cell surface causing both increased permeability to substances that normally cannot enter and a significant loss of LPS (Leive 1965b). In fact, 30 to 50% of LPS, 5 to 10% protein and 5% phospholipid are released during EDTA treatment. Because of the increased permeability due to EDTA treatment and the subsequent centrifugation of the MVs, twice for 40 min at 30,000 × g, it seems possible that the DNA contained within the MVs would leak out into the supernatant or would stick to the outside of the membranes. Thus, following DNase treatment of MVs, the free and outer surface-associated DNA would be susceptible to DNase activity. Leive (1965a) reported that there was a negligible loss of low molecular weight cell components such as nucleotides and amino

acids from the cells, but MVs do not contain intact PG or PM that would possibly stop DNA from escaping. Accordingly, EDTA could cause the leakage of DNA from the vesicles and thus the presence of DNA within O157:H7 MVs is surprising.

MV-DNA was further analyzed by PCR with primers made to amplify regions of virulence genes *eae*, *stx1*, *stx2* and a regulatory gene *uidA*. Here, the presence of these genes was observed with intact vesicles treated with DNase or untreated. Although amplification of these gene products in the MV samples was observed, this could be due to MVs alone or a result of contaminating parent cells in the preparation. PCR involves the amplification of a selected DNA sequence so that it is increased exponentially, where even a single gene copy can be amplified to a million copies within a few hours of PCR (Atlas and Bej 1994). Thus, even if only one cell is present, the genes targeted above will be amplified, resulting in the over representation of the any DNA present in MVs.

Furthermore, in DNA transfer experiments both noncompetent and competent *E. coli* JM109 cells were prepared and analyzed for their uptake of *E. coli* O157:H7 MV-DNA (Kolling and Matthews 1999). Five colonies of the assumed transformants were randomly selected and analyzed by PCR to determine the presence of *eae*, *stx1* and *stx2*. It was concluded that due to the amplification of these genes by PCR, regardless of whether the recipient strain was competent, the transfer of genetic material did occur. It is striking to note that the colonies studied were not tested to differentiate whether these transformants were actually *E. coli* JM109 and not the donor cells, *E. coli* O157:H7. Considering that the resulting transformants were not plated on selective media and the cells were not treated in any way to remove the MVs, it is possible that the donor MVs remain attached to the recipient cells. Even if the transformation of the recipient strain

was not successful, a PCR product would still result due to either the existing donor strain or the attached MVs containing O157:H7 DNA.

Following this first investigation of *E. coli* O157:H7 MVs, a continuation of this study was done to further clarify the different origins of the DNA present in O157:H7 MVs and to examine whether this genetic material could be transferred (Yaron et al. 2000). Again, these researchers isolated MVs by the protocol designed by Kadurugamuwa and Beveridge (1995) and also terminated DNase activity by EDTA treatment. PCR was performed on DNase-treated MVs and purified vesicle DNA using specific primers designed for genes found on regions of chromosome, phage and plasmid DNA. PCR amplification of genes encoded on the putative cryptic prophage, the chromosome and the 3.3 kb and 92 kb plasmids were observed using purified and concentrated (10-fold) MV-DNA. The MV-DNA was isolated by treating the MVs with DNase, lysing them with 0.125 % Triton X-100 and purifying the DNA using a glass fiber matrix-binding and elution technique. It is interesting that such a concentrated amount (10-fold) of DNA was required to observe a PCR product for these genes. The need for such a large amount of purified MV-DNA may not necessarily be due to the low presence of DNA for these genes in the MVs as stated by Yaron et al. (2000), but could be due to the contribution of DNA from few contaminating parent cells present in the MV preparation, especially when a 10-fold MV suspension was used for DNA isolation. Genes found on plasmid pO157 and temperate bacteriophage DNA were detected by PCR using non-concentrated MV-DNA. Because the amplification of these genes used non-concentrated DNA, it was suggested that phage 933W and plasmid pO157 are more

commonly associated with MVs, since they were detected consistently without using concentrated vesicle DNA.

This analysis concluded that MVs of O157:H7 harbored genes of different origin; i.e., those being chromosomal, phage and plasmid. Although this may be true, it is difficult to conclude this with such certainty considering that any DNA from whole cells, especially in the concentrated MV-DNA samples could contribute to visualization of the PCR product.

It is apparent that both *E. coli* MV-DNA studies raise questions concerning the true relationship of MVs with DNA. Additional experiments examining the role of *E. coli* MVs in transformation have also been attempted and evoke additional concerns regarding the reliability of the experimental procedures and the results obtained. This information will be discussed in further detail in Chapter 3.

As described in Chapter 1, only a few studies have explored the association of DNA with MVs of *P. aeruginosa*. These experiments examined MVs obtained from the MV isolation procedure of Kadurugamuwa and Beveridge (1995). Therefore, further work is required with the use of MVs prepared from a cell-free MV isolation procedure in order to verify these findings.

Thus far, MV-DNA research is not conclusive and requires more careful and extensive characterization. There is an obvious concern with the absolute purity of isolated MVs (i.e., free from bacterial and contaminating DNA). Before any conclusions can be made regarding the presence of DNA in MVs and its possible function, it is crucial that an MV isolation protocol be developed that ensures MV samples are completely cell- and external DNA-free. Once such a system is established, results

obtained from a continuation of this work would dispel any uncertainty that exists with the previous findings. The work would reflect the true nature of MV-DNA without any contribution made by the possibility of remaining whole cells.

2.1.1 Present Study

In the first part of this study using the original protocol (Kadurugamuwa and Beveridge 1995) for isolating MVs an MV preparation was analyzed for contaminating whole cells by EM and surface plating on LB plates. The contaminating whole cells were identified using basic systematic bacteriology i.e., Gram-staining, wet mounts, oxidase and catalase tests. The goal of this was to eliminate cellular contamination by establishing a stringent MV isolation procedure. Once this cell-free MV system was designed the second part of this study examined the DNA content of MVs by fluorometric analysis and characterized this DNA by PCR. The objective of this work was to understand the true nature of MV-DNA association based on MVs alone.

2.2 Materials and Methods

2.2.1 Bacterial strains and culture conditions

Competent *P. aeruginosa* PAO1 serotype O5 was prepared by cold shock of $MgCl_2$-treated cells and was transformed (Irani and Rowe 1997) with plasmid pAK1900 isolated from *E. coli*/pAK1900. pAK1900 (Fig. 4) is an *E. coli-P. aeruginosa* shuttle cloning vector and contains a β-lactamase gene (encodes for ampicillin and carbenicillin resistance) (Jansons et al. 1994). A restriction digest with *Hpa*I and *Bam*HI was performed on plasmid DNA isolated from PAO1/pAK1900 to ensure that transformants did contain pAK1900. PAO1 and PAO1/pAK1900 were grown in Trypticase soy broth (TSB) (BBL Laboratories) to late exponential phase (~10^8 CFU/ml) on an orbital shaker (125 rpm) at 37°C. To select for the presence of PAO1/pAK1900, 500 μg/ml of carbenicillin (Sigma Chemical Co., St Louis, Mo.) was added to the culture medium.

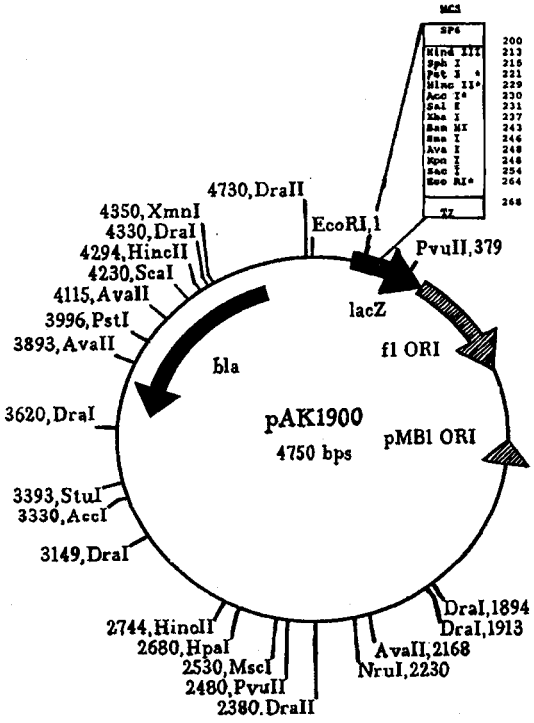

Fig. 4. Plasmid pAK1900

2.2.2 Original MV isolation procedure

MVs from PAO1 and PAO1/pAK1900 was isolated according to the protocol of Kadurugamuwa and Beveridge (1995). Briefly, *P. aeruginosa* was grown in 1 L of TSB to late exponential growth phase at 37°C on an orbital shaker with an agitation rate of 125 rpm. Whole cells were removed by centrifugation for 20 min at 6,000 × g in a Sorval GS3 or SLA3000 rotor (Sorval Products, L.P., Newtown, CT). The supernatant was filtered once through 0.45 and 0.22 μm cellulose acetate membranes (Osmonics, Minnetonka, MN) to remove whole cells. 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 Inc., Toronto, ON). The supernatant was carefully aspirated from the tubes and the vesicle pellet was resuspended in the small amount of remaining supernatant, collected in Eppendorf tubes and centrifuged at 16,000 × g for 30 min in a benchtop centrifuge to concentrate the MV pellet. The pellet was washed in 50 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES, pH 6.8) (ICN Biomedicals Inc., Aurora, OH), centrifuged at 16,000 × g for 30 min, resuspended in 50 μl of HEPES, and stored at 4°C until ready to use that same day. MVs were examined by EM and aliquots of the MV preparation were plated on Luria Bertani (LB) agar and incubated overnight at 37°C to observe any colony growth.

2.2.3 EM

Negative staining of MV preparations was performed to observe the purity of the MV samples and in later experiments to verify MV lysis. A 10 μl sample was placed on a 200-mesh carbon- and Formvar-coated nickel grid (Marivac) and stained with aqueous 2% (wt/vol) uranyl acetate (Kadurugamuwa and Beveridge 1995). The grids were

examined with a Philips EM300 transmission electron microscope (TEM) operating under standard conditions at 60 kV with the liquid nitrogen cold finger in position. A thin frozen film of an MV preparation was also prepared and cryo-TEM examinations were carried out. This method will be described in more detail in Chapter 4.

2.2.4 Identification of contaminants

Gram staining, wet mounts, oxidase and catalase tests were performed on the contaminating bacteria that accompanied the MV preparations. Colonies were also grown on Centrimide agar (BBL Laboratories) to further determine whether the contaminant was the MV-producing strain *P. aeruginosa.* Centrimide (cetyltrimethylammonium bromide), is cationic detergent that causes the release of nitrogen and phosphorus from the bacterial cell. This germicidal activity does not affect *P. aeruginosa* (Lowbury and Collins 1955).

2.2.5 Modified isolation procedure of MVs

PAO1 and PAO1/pAK1900 MVs were isolated by the protocol originally designed by Kadurugamuwa and Beveridge (1995) with modifications to ensure complete purity of the MV sample. Following centrifugation of the bacterial culture and removal of the cell pellet, the supernatant was sequentially filtered three times through 0.45 and 0.22 μm cellulose acetate membranes to remove remaining cells. Ultracentrifuge tubes were presoaked in a sterilizing agent (1/100 dilution of Sani Rinse; Wil-Don, Kitchener, ON) for 30 min and rinsed three times in sterilized nanopure water to remove residual detergent. MVs were recovered from the resulting filtrates, washed and stored in HEPES buffer, as indicated above. The purity of the MV sample was examined by plating of aliquots on LB plates and incubation at 37°C overnight to allow for colony growth.

MV protein content was determined using a Micro BCA protein assay reagent kit (Pierce, Rockford, IL).

2.2.6 Fluorometric quantification of DNA

Surface-associated and DNA contained within MVs was quantified by the PicoGreen assay (Molecular Probes, Eugene, OR). A 20 μg protein sample from n-MVs or p-MVs was lysed with GES reagent (5 M GES, 100 mM EDTA, 0.5% (vol/vol) Sarkosyl) to release DNA from MVs. Experiments were also performed with MVs that were pre-treated with pancreatic DNase I (50 μg/ml (Sigma) for 1 h at 37°C) to digest any DNA bound to the outer surface of MVs after which they were washed and then lysed with GES reagent. Samples were further processed according to the manufacturer's instructions and fluorescence was measured in a Hitachi F-2000 fluorescence spectrophotometer.

2.2.7 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 X PCR buffer (Roche), 1.5 mM $MgSO_4$, nucleotide-free water and 2.5 U *Pwo* polymerase (Roche). DNA primers used for the amplification of the β-galactosidase (*lac*) gene on pAK1900 were pAKF (5′-GACCATGATTACGCCAAGCT-3′) and pAKR (5′-TTTGGGGTCGAGGTGCCG-3′), to produce a PCR product of 475 bp. Primers designed for the chromosome encoded *oprL* gene, were oprLF (5′-TGACGGTCGCCAACGGTTAC-3′) and oprLR, (5′-CTGGAGCTGCATGAACAGTT-3′) to produce a PCR product of 760 bp. DNA from a 10 μl sample of untreated MVs or DNase-treated MVs was used as template. A 1 μl aliquot of a PAO1 whole-cell suspension and purified 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 + 5 s per 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.

2.3 Results

2.3.1 Identification of contaminant growth in MV preparations

Following the isolation of MVs using the original isolation procedure, the purity of the MV sample was examined for contaminating cells. An overnight incubation of isolated MVs on LB plates resulted in 5.89 ± 1.23 colonies per MV run (500 ml original volume). Visualization of the MV preparation using thin frozen foils by cryo-TEM identified the presence of whole cells. In Fig. 5, it can be seen that the contaminating cell is likely PAO1 since it was a rod-shaped bacterium (of appropriate dimensions), with MVs visible in the surrounding background.

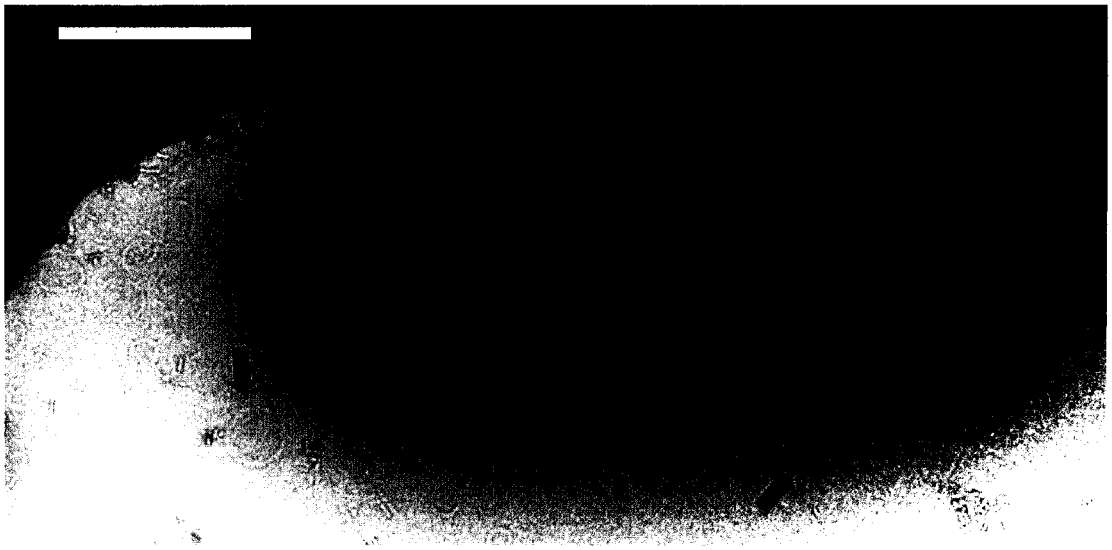

Fig. 5. Thin frozen foil of an MV preparation obtained from the original MV isolation procedure. Arrows point to MVs surrounding a contaminant bacterial cell. See Chapter 4 for more cryo-TEM details of MVs as well as the methodology involved. The scale bar represents 500 nm.

2.3.2 Identification of Contaminant

Once contaminating cells were plated and grown, they were Gram-stained, tested for oxidase and catalase and grown on Centrimide agar to identify the contaminant. The contaminant was a motile, Gram-negative rod and scored positive for both catalase and oxidase production. Bacterial growth and pigment development were seen on Centrimide agar plates. Also, pyocyanin, a pigment produced by *P. aeruginosa* was observed as a characteristic strong blue-green pigment throughout the medium. This data strongly suggested that the contaminant was *P. aeruginosa* and was presumed to be the parent strain PAO1 or PAO1/pAK1900.

2.3.3 Development of a cell-free MV isolation procedure

It was concluded that some of the contamination was due to the presence of cells residing on the polycarbonate tubes (Beckman) used in ultracentrifugation of the supernatant. Because these tubes can withstand only limited autoclaving, other agents were tested to reduce and discourage bacterial growth. Hydrogen peroxide and other detergents were used but gas treatment (30 h incubation with ethylene oxide) was most effective in contaminant reduction. This method, although most effective, resulted in the reappearance of cells after only one round of MV isolation. The modified MV procedure (extra filtration and soaking tubes in detergent) allowed for isolation of MVs that were absent of *P. aeruginosa* cells as observed by TEM (Fig 6A) and by lack of growth on LB plates (data not shown). Thus, this procedure was successfully designed to eliminate any whole cells that were previously found to accompany MV preparations.

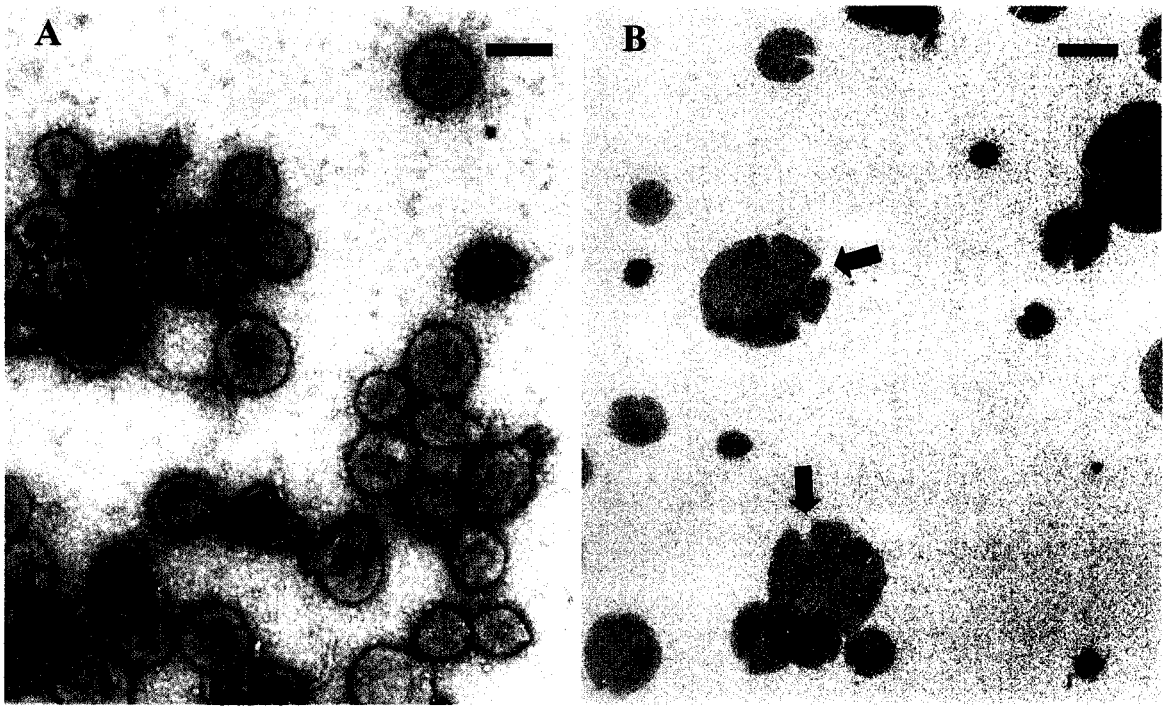

Fig. 6. Negatively stained p-MVs observed by TEM. (A) Isolated fraction of p-MVs. Note the absence of cells in the sample that were prepared from the modified isolation procedure. (B) GES-treated MVs. Arrows point to areas of MV breakage. The scale bars represent 100 nm.

2.3.4 Confirmation that MVs contained intact DNA

PAO1 was transformed with plasmid pAK1900 so that MVs (i.e., p-MVs) isolated from this strain could be analyzed for the possible incorporation of plasmid DNA. DNA must first be liberated from vesicles for accurate fluorometric detection. GES was used to solubilize MVs and visualization of MVs by TEM showed that MVs were broken (Fig. 6B). 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. A quantity of 0.31 ng DNA/20 μg MV protein was found associated with n-MVs (Table 1) which confirmed previous results of Kadurugamuwa and Beveridge (1995). Most of this DNA was contained in the n-MVs and not surface associated since ~70% of DNA content remained following DNase treatment. p-MVs contained ~25 × more DNA but only one half of this was inside the MVs (Table 1). This DNA was presumably associated with the MV surface.

2.3.5 Determination that 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 detectable product was produced after DNase treatment of intact MVs (Fig. 7A). Using DNA from 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. 7B).

As with the amplification of *oprL*, following DNase treatment digested surface associated chromosomal DNA from the MVs.

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

Strain	Vesicle Type	ng of DNA/20 μg of MV protein (avg ± SD)[a]
PAO1	n-MV	0.31 ± 0.17
PAO1 DNase treated	n-MV	0.23 ± 0.10
PAO1/pAK1900	p-MV	7.80 ± 0.41
PAO1/pAK1900 DNase treated	p-MV	3.91 ± 0.31

MVs lysed with GES reagent.
[a] $n = 3$

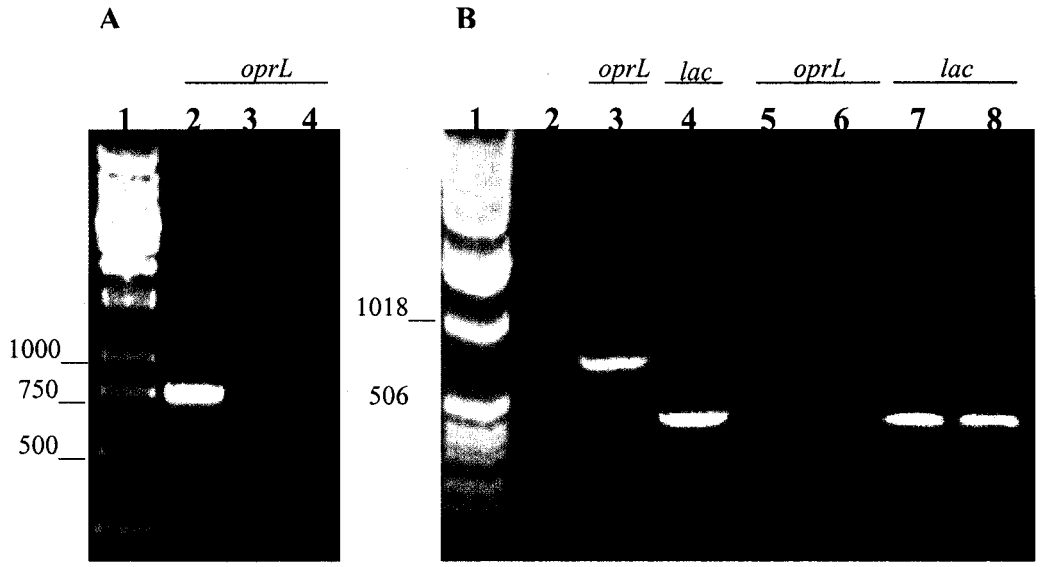

Fig 7. PCR analysis to determine the presence of chromosomal and plasmid DNA associated with MVs. A. PCR amplification of n-MVs, using primers specific for *oprL* (760 bp). Lane 1, 1 kb DNA ladder; lane 2, PAO1 genomic DNA (control); lane 3, n-MVs; lane 4, DNase-treated n-MVs. B. PCR amplification of p-MVs, using primers specific for *oprL* and *lac* (475 bp). Lane 1, 1 kb DNA ladder; lane 2, no template (control); lane 3, PAO1 genomic DNA (control); lane 4, plasmid pAK1900 (control); lanes 5 and 7 p-MVs; lanes 6 and 8, DNase-treated p-MVs. Molecular sizes in bp are as indicated on the left of each panel.

2.4 Discussion

Through the use of the original MV isolation protocol, the appearance and persistence of contaminant cells tainted the purity of MV suspensions. The observation of whole cells in the MV preparations by EM and colony growth on nutrient agar plates indicated that during the isolation procedure, cells remained. Only one cell type was evident and with simple identification techniques, the bacterial contaminant was identified as *P. aeruginosa* and presumed to be the parent PAO1 or PAO1/pAK1900 strain. Because the ultracentrifuge tubes used in the final stages of MV isolation cannot withstand autoclaving, the presence of whole cells could partly be due to their presence on the tube walls. The use of hydrogen peroxide and other detergents did not seem to discourage the growth of bacteria while gassing the tubes with ethylene oxide did. This procedure required longer time and is expensive especially since following the use of these tubes after only one MV isolation run, gassing was again required due to the re-contamination of the tubes and thus the MV sample. It was evident that a more stringent protocol was needed and thus a modified procedure based on Kadurugamuwa and Beveridge's original protocol (1995) was designed. Additional filtration of the supernatant (three times through 0.45 and 0.22 μm cellulose membranes) and pre-soaking of the ultracentrifuge tubes in Sani-rinse detergent for a half hour eliminated the persistence of cell contaminants.

There are several implications on other studies now that we have derived an appropriate cell-free MV isolation system. Other MV research which quantitated periplasmic components, such as toxins and enzymes within MVs, could be inaccurate. If the enzyme activity is measured based on a certain amount of MV protein and if whole

cells are present, the protein content of the cells will be a factor. In bactericidal experiments the secretion of cellular enzymes (i.e., protease, elastase, collagenase and hemolysin) by bacteria that are toxic to the host could contribute to cell death of the target organism. Thus, in such an experiment, the measurement of the CFU would either over-represent (by bactericidal activity of the contaminating cells) or under-represent (by the presence of the cells and growth on plates) the actual MV-killing. Lastly, research examining the potential of MVs from *P. aeruginosa, S. flexneri, B. pertussis, H. felis, H. pylori* and *N. meningiditis* (Fu et al. 2000; Hozbor et al. 1999; Kadurugamuwa and Beveridge 1999; Keenan et al. 1995; 1998; 2000a, 2000b; Mirlashari et al. 2001) as possible vaccine delivery systems must be re-examined to ensure the removal of all cells. This would ensure that an MV-vaccine is safer due the reduction of endotoxic LPS. Therefore, MV-vaccine research can benefit with the development of a cell-free MV isolation system.

Because the isolation of MVs was designed to ensure no cellular contamination from the parent MV-producing strain, the work done to quantify DNA was not over-estimated and the characterization of the type of DNA was not misrepresented. Here DNA was found to be associated with n-MVs and p-MVs from PAO1 and PAO1/pAK1900, respectively.

A comparison of the fluorometric DNA quantification values obtained in previous studies can be made to examine the difference in MV-DNA content between strains of the same species. For example, *E. coli* O157:H7 ATCC MVs contained ~3 × less DNA than MVs from *E. coli* O157:H7 8302 (Kolling and Matthews 1999), and *P. aeruginosa* H103 MVs contained ~2 × less DNA than MVs from *P. aeruginosa* ATCC 19660

(Kadurugamuwa and Beveridge 1995). Values obtained for PAO1 n-MVs in this study were comparable (~1.5 × difference) to DNA content of the previously determined MVs from *P. aeruginosa* strains (ATCC 19660 and H103), while p-MVs contained a far greater amount of DNA (~20 - 35 × more) compared to these three n-MV-producing strains. When comparing DNA content of MVs from species to species, depending on the strain, n-MVs of *P. aeruginosa* ATCC had a range of ~5 - 22 × less MV-DNA to that from *E. coli* O157:H7 strains. p-MVs on the other hand contained a similar range of DNA content, ~2 - 5 × more, compared to those from *E. coli*. It appears from this data that variable amounts of MV-associated DNA exist between different MV-producing strains with even more variation detected on a species to species level. It is unclear why such variation in DNA uptake would result and whether the amount of encapsulation influences a specific function.

Furthermore, PCR revealed that chromosomal DNA was found only externally associated with n- and p-MVs, without any inside or protected by the MVs. In addition, p-MVs revealed plasmid DNA to be both externally and internally associated. These findings are most similar to the *Neisseria* system where both chromosomal and plasmid DNA were found on the outer surface of the MVs and with only plasmid DNA found within the MVs (Dorward et al. 1989). In contrast, *E. coli* O157:H7 MVs contained DNA with genes associated with chromosomal, phage and plasmid origins (Yaron et al. 2000).

It is remarkable that DNA is packaged into MVs of *Escherichia*, *Haemophilus*, *Neisseria* and *Pseudomonas* species (Kahn et al. 1982; 1983; Dorward and Garon 1989; Dorward et al. 1989; Kadurugamuwa and Beveridge 1995; Kolling and Matthews 1999;

Yaron et al. 2000), since this process must be complex. For these bacteria, not only are extrachromosomal elements packaged but also chromosomal DNA. Somehow plasmid and bacteriophage DNA target themselves for packaging and even discrete lengths of genomic DNA also become internalized within MVs. Although it is unclear as to whether the incorporation of DNA within an MV is a random or a regulated event, it has been suggested that in the *Neisseria* MV system, nucleic acid incorporation is regulated due to the appearance of more DNA than RNA within the vesicles (Dorward et al. 1989). Contrary to this observation, researchers working with *E. coli* O157:H7 MVs suggested that because virulence genes along with a regulatory gene (*uidA*; found in ~95% of all *E. coli* isolates) were found contained within MVs, internalization of DNA is random (Kolling and Matthews 1999). These two conclusions are based on little experimental evidence, where the amount or the type of genetic material packaged does not warrant the assumption that MV internalization of genetic material is either under genetic regulation or randomly incorporated.

This experimentation has shown that plasmid DNA is incorporated within MVs from PAO1/pAK1900. The next step is to explore whether this DNA can be transported to other cells via MVs that could result in genetic transformation of the recipient cell.

3.0 INVESTIGATION OF THE TRANSFORMATION POTENTIAL OF p-MVS

3.1 Background

Horizontal gene transfer is a process whereby genes are acquired from different genetic backgrounds. This process has been implicated as an important adaptive mechanism that gives bacteria increased genetic variation (Dahlberg et al. 1998). The importance of horizontal gene transfer is evident when bacterial populations encounter strong selective pressures to alter their innate traits; for example, when they are subjected to antibiotics or toxic pollutants. Such pressures may have caused the dissemination of genes or gene clusters responsible for antibiotic resistance and catabolism of xenobiotics (Mazel and Davies 1999). Consequently, speciation and sub-speciation in bacteria is presumed to be a result of these adaptive events (de la Cruz and Davies 2000). Acquired genes are transferred on mobile elements such as plasmids and transposons that can be delivered to bacteria by three distinct methods; conjugation, transduction, and transformation. Although all three mechanisms have been shown to occur in nature, for the purpose of this study, only transformation will be described. Transformation is a process by which bacteria take up naked exogenous DNA (plasmid or chromosomal) thereby acquiring an altered genotype that is heritable (Smith et al. 1981). Because transformation does not require cell to cell interactions, it has been suggested that it may represent the most important mechanism of DNA acquisition between distantly related bacteria (Mazodier and Davis 1991). There are certain limitations in transformation. Because the DNA is naked, it is susceptible to enzymatic and chemical degradation. Also, the recipient cell must be competent, the genetically programmed property that renders bacteria capable of binding and taking up DNA (Stewart and Carlson 1986).

The steps involved in transformation are: 1) development of competence, 2) binding of DNA, 3) entry of DNA, and 4) intracellular processing of DNA (i.e., when DNA is integrated by recombination into the chromosome or when an autonomous plasmid is stably established) (Mercenier and Chassy 1988; Lorenz and Wackernagel 1994). Although this process involves a few seemingly simple steps, there are only a limited number of bacterial species that are naturally competent. The species that lack this characteristic require methods to artificially render them competent. This is accomplished by manipulation of the bacteria chemically (i.e., calcium/magnesium chlorides), physically (i.e., electroporation, freeze thaw) or enzymatically (i.e., using muraminidases and peptidases) causing the formation of spheroplasts or protoplasts, which allows entry of the transforming DNA (Lorenz and Wackernagel 1994).

With these obstacles in mind, could MVs circumvent the transformation barriers of nuclease degradation and competence? It has been shown that DNA can be present within MVs. This association could represent a unique and ideal strategy of natural transformation where naked DNA is protected from exonucleases by a membrane. Also, competence would not be compulsory, at least not for initial binding to the OM and transport of DNA into the recipient's periplasm. This is because MVs have been shown to readily fuse to the OM of Gram-negative bacteria (Kadurugamuwa and Beveridge 1996; 1999). It is by this way that foreign autolysins and β-lactamases can be delivered to other bacteria (Ciofu et al. 2000; Li et al. 1996; 1998; Kadurugamuwa et al. 1998; MacDonald and Beveridge 2002). Once in the periplasm DNA would need only to transfuse through the recipient's PM and be integrated into the genome for successful transformation. This mechanism of MV-aided transformation could increase the

efficiency of natural transformation, which has been shown in laboratory studies to have frequencies often below 10^{-10} (Jeffrey et al. 1990).

MV-mediated DNA transfer has been examined in *N. gonorrhoeae*, *E. coli*, *H. influenzae and H. parainfluenzae* (Dorward et al. 1989; Kahn et al. 1983; Kolling and Matthews 1999; Yaron et al. 2000). To determine the role of *N. gonorrhoeae* MVs in transformation, MVs from gonococcal strains containing different forms of antibiotic resistance genes (i.e., plasmid or chromosomal) were examined (Dorward et al. 1989). MVs from strain 31426, which carries a penicillinase-specific R-plasmid (*bla*$^+$), incubated with wild-type JS1 and DNase resulted in recipient cells acquiring penicillin resistance at a transformation frequency of 6.5×10^{-3} (DNA marker transformants/viable cell). Transformed cells were able to grow in the presence of antibiotic by producing penicillinase. JS1 cells incubated with MVs from strain Dst11, a streptomycin-resistant (Str^R) transformant (chromosomally encoded) of JS1, were not transformed to Str^R. Strain FA589 (*bla*$^+$, Str^R) MVs incubated with JS1 resulted in JS1 cells expressing the *bla*$^+$ property, but recipient clones which were Str^R were not recovered. As well, conjugative plasmids, absent in JS1, were also expressed in all recipient clones, although it was not clear whether conjugative and R-plasmids were transferred together or separately. The transfer of only R-plasmid and not chromosomal MV-DNA to recipient cells is consistent with the previous observation that only plasmid DNA is protected within MVs. These data suggest that MVs can effectively carry and transfer plasmid DNA which may serve as a mode of genetic exchange for *N. gonorrhoeae*.

As mentioned in the previous chapter, MVs from *E. coli* O157:H7 delivered virulence genes to *E. coli* JM109 (Kolling and Matthews 1999). Although the authors

suggested that *E. coli* MVs acted as vectors of DNA transport, there are concerns with the validity of the experimentation. Further studies by Yaron et al. (2000) re-examined the potential of O157:H7 MVs to transfer DNA to JM109 and again similar issues with the reliability of the results are a concern. Following incubation of JM109 cells in nutrient broth with O157:H7 MVs and DNase treatment, the suspension was centrifuged and an aliquot of the pellet was removed for PCR detection of O157:H7 genes (*stx2, hlyCA* and endpoints of 933W phage). A PCR product was observed for 933W, *stx2* and *hlyCA*, suggesting that O157:H7 MVs successfully transferred the encapsulated genetic material to JM109. This conclusion is bold since centrifugation of the sample could have pelleted some MVs which were attached to the cells. Therefore, removal of a pellet sample following centrifugation and detecting the selected genes by PCR does not represent an accurate method in the determination of successful introduction of DNA into the recipient cells, since those cells with attached MVs would produce a positive PCR result.

To further demonstrate that this observed introduction of DNA was MV-mediated, Yaron et al. (2000) repeated these experiments using MVs that were treated with lysozyme. The attempt to lyse MVs by treatment with lysozyme is an odd choice. Lysozyme should not lyse MVs because it would have no hydrolytic effect. Lysozyme acts by hydrolyzing the $\beta(1\rightarrow4)$ glycosidic linkages between N-acetylmuramic acid to N-acetylglucosamine, the polysaccharide component of PG (Voet and Voet 1995). MVs do not contain intact PG and even if PG was an MV component, in order for this enzyme to be active it requires access to these sites. This would only occur if the membrane was destabilized by the addition of detergents or by chelators (Witholt et al. 1976). This is because lysozyme, approximately 14 000 Da, would be unable to penetrate the *E. coli*

membrane which has an exclusion limit of 600 to 800 Da (Benz 1985). Because lysozyme treated MVs were used as a negative control for the above transformation experiments, I find it suspicious that transformants were not observed, since the MVs would not be lysed by this method.

Other transformation experiments in this same study found that MVs of *E. coli* O157:H7/pGFP (i.e., a strain containing a plasmid that encodes for Amp^R and GFP) transferred pGFP to JM109 cells, producing Amp^R colonies that fluoresced when exposed to UV light. This event occurred at a transformation frequency of 3×10^{-10}, a value insignificantly lower than that of MV-mediated transformation of *N. gonorrhoeae.* Lastly, Vero cells were incubated with filtered supernatants of O157:H7 MV-transformed JM109 and *Salmonella enteritidis* suspensions. The cytotoxicity of these supernatants on the Vero cells was measured to determine the level of expressed virulence genes. The supernatants of transformed cells were more cytotoxic than the untransformed cell supernatants of the same species, suggesting that Stx was expressed by the former strains. MVs were less cytotoxic than supernatants of transformed cells, eliminating the possibility that MVs were solely responsible for cytotoxicity. Based on the results obtained by this study, genes encoding virulence and antibiotic resistance were deemed to be transferred by O157:H7 MVs.

As mentioned in Chapter 1, *H. influenzae* and *H. parainfluenzae* form MVs called transformasomes during competence development. Unlike MVs from other Gram-negative bacteria, transformasomes are not released into the external milieu but remain attached to the cell surface. DNA uptake studies with transformasomes suggest that these structures contribute to *Haemophilus* transformation. A model describing this

mechanism has been developed and is distinct from the *Neisseria* and *Escherichia* MV-DNA delivery system described above. Here, DNA that exists in the extracellular environment is taken up by the transformasome by an unknown method and is protected from restriction enzymes and DNase (Kahn et al. 1982; 1983). In the case of *H. influenzae*, if the DNA is linear, one end of the DNA exits from the transformasome into the cell, is degraded and searches for homologous regions on the chromosome (Barany et al. 1983). Once homology is found, the undegraded linear strand remaining in the transformasome exits and becomes integrated in the chromosome (Barany and Kahn 1985). If the genetic material within the transformasome is covalently closed circular DNA, it is unable to exit the transformasome and thus can not be further processed (Kahn et al. 1983). This occurrence may explain the low frequency of plasmid transformation in *H. influenzae*.

To date, several studies provide evidence to support the contribution of MVs in transformation. Whether p-MVs of PAO1/pAK1900 also participate in this alternate mechanism of genetic transfer will be examined in this current study. Again it must be re-emphasized that this experimentation must use MVs that are 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.

3.1.1 Present Study

With the presence of pAK1900 within MVs, the objective of this study was to explore the involvement of p-MVs as DNA delivery agents. Time course p-MV transformation experiments with PAO1 and *E. coli* DH5α as the recipients in nutrient

rich (LB and SOC) media and under nutrient limited conditions (HEPES buffer) were performed to determine if transformation had occurred.

3.2 Materials and Methods

3.2.1 Bacterial strains and culture conditions

PAO1 and *E. coli* DH5α were grown in TSB to late exponential phase (~10^8 CFU/ml) on an orbital shaker (125 rpm) at 37°C.

3.2.2 Transformation experiments

Transformation experiments were performed using p-MVs with either PAO1 or DH5α as the recipients. DNase was added to treated MV preparations to degrade exogenous DNA.

Assay 1. Recipient cells were grown to exponential phase and resuspended in fresh LB to a concentration of 10^9-10^{11} cells/ml. A 900 μl sample of recipient cells was added to 100 μl of MVs (100 μg/ml) with or without 1 μg/ml DNase I. This suspension was incubated at 37°C with agitation (125 rpm). At 1 and 20 h, a 100 μl sample was plated on LB/carbenicillin (500 μg/ml) plates and incubated overnight at 37°C. Colonies on plates were deemed to be successful transformants.

Assay 2. This followed the protocol of Yaron et al. (2000) with some modifications. Recipient cells were grown to exponential phase and resuspended in cold SOC medium to a concentration of 10^9-10^{11} cells/ml. An MV sample (100 μg/ml protein), 1 μg/ml DNase I, the cell suspension (100 μl) and 800 μl of SOC were combined and incubated at 37°C statically for 1 h followed by 2 h with shaking (125 rpm). Ten ml of LB were added to this suspension and incubated for 20 h with shaking. Aliquots of this suspension (100 μl) at 2, 4, 8, 12 and 20 h were plated on LB/carbenicillin and incubated overnight at 37°C.

Assay 3. Experimentation was followed as in assay 2 but with various media (LB, HEPES + LB and HEPES).

The same experimental controls were followed for all protocols; i.e., n-MVs or 1 μg/ml of purified pAK1900 DNA in place of p-MVs under the same transformation conditions, MVs spread directly on LB plates, and PAO1 and *E. coli* DH5α spread on LB and LB/carbenicillin plates.

3.3 Results

It was expected that DNA contained within MVs would be protected from exonucleases, thereby increasing the efficiency of DNA delivery and uptake into a recipient cell. p-MVs did not have the ability to transform PAO1 nor DH5α. Time course p-MV transformation experiments of 1 to 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 for all three of the assays (Table 2).

The experimental controls confirmed that: 1) pAK1900 can replicate in PAO1 and DH5α 2) n-MVs or purified pAK1900 did not produce transformants, 3) donor MVs did not produce colonies on LB plates, and 4) recipient cells grew on LB plates but did not grow on LB/carbenicillin plates.

Table 2. Transfer of genetic material by MVs isolated from PAO1 pAK1900

Assay [a]	Media	Recipient cell	DNase	Transformants				
				2 h	4 h	8 h	12 h	20 h
1	LB	PAO1	-	0	0	0	0	0
	LB	PAO1	+	0	0	0	0	0
	LB	DH5α	-	0	0	0	0	0
	LB	DH5α	+	0	0	0	0	0
2	SOC/LB	PAO1	-	0	0	0	0	0
	SOC/LB	PAO1	+	0	0	0	0	0
	SOC/LB	DH5α	-	0	0	0	0	0
	SOC/LB	DH5α	+	0	0	0	0	0
3	LB/LB	PAO1	-	0	0	0	0	0
	LB/LB	PAO1	+	0	0	0	0	0
	LB/LB	DH5α	-	0	0	0	0	0
	LB/LB	DH5α	+	0	0	0	0	0
	HEPES/LB	PAO1	-	0	0	0	0	0
	HEPES/LB	PAO1	+	0	0	0	0	0
	HEPES/LB	DH5α	-	0	0	0	0	0
	HEPES/LB	DH5α	+	0	0	0	0	0
	HEPES/HEPES	PAO1	-	0	0	0	0	0
	HEPES/HEPES	PAO1	+	0	0	0	0	0
	HEPES/HEPES	DH5α	-	0	0	0	0	0
	HEPES/HEPES	DH5α	+	0	0	0	0	0

[a] All assays were done in triplicate.

3.4 Discussion

Because MVs released into the external environment can attach, fuse, and deliver internalized toxins and enzymes to bacteria, it was anticipated that DNA within MVs could also be transported by this mechanism (Kadurugamuwa and Beveridge, 1996; 1998; 1999). DNA, once delivered to the periplasm, 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 genetic material would be passed from one bacterium to another via a protective membrane without the need for competence. Although this system is intuitively logical, the experimentation reported here failed to demonstrate DNA transfer via MVs.

These 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 are not endowed with transformation capability. It is also possible that the high stringency used here for MV isolation and purity was not achieved by these other studies.

It can be assumed that p-MVs successfully delivered DNA to the periplasm of recipient cells. Here, the MV autolysin could hydrolyze PG allowing the DNA to contact the PM. Thus, the only remaining barrier to pAK1900 for transport into the cytoplasm would be the PM. In these present experiments pAK1900 could not by-pass this membrane. This could be due to lack of a transport or destructive degradation of the plasmid during transport. Because plasmid DNA utilizes the same natural transformation

process for linear DNA, its survival inside the cell may be difficult. The mechanism for DNA uptake involves binding and cleaving of double-stranded DNA and transport of one strand across the membrane while the other strand is degraded and released into the medium (Dubnau 1999; Dubnau and Provvedi 2000; Smith et al. 1981). This poses difficulty for supercoiled DNA such as pAK1900 where fragmentation may have occurred beyond its ability to function.

There are other factors that could have attributed to the lack of transformation via p-MVs. Generally, the occurrence of natural transformation requires the fine-tuning of several conditions including the composition of the culture medium, temperature, pH, etc. which can differ depending on the species. The supplementation of divalent cations in the nutrient broth is necessary for uptake but not binding of DNA in *Acinetobacter calcoaceticus* (Lorenz et al. 1992). In *B. subtilis* uptake requires Mg^{2+} and by increasing the concentration of Mg^{2+} from 0.1 to 10 mM the number of transformants increases by 100-fold (Garcia et al. 1978). Temperature could also be a factor. In *Vibrio* species transformation drops 2-fold when the temperature is raised from 33°C to 37°C (Frischer et al. 1993), even though growth is best at 37°C. *Azotobacter vinelandii* and *Pseudomonas stutzeri* grown at various pHs are transformable at pH 7 with a drastic decline as the pH either increases or decreases (Lorenz and Wackernagel 1991; Page 1982). Although these examples describe the requirements of transformation for competent bacteria, these examples clearly emphasize that specific conditions are required for successful DNA transfer, which further demonstrates the complexity of this genetic transfer event.

Is the internalization of DNA by MVs really that important for protection and transfer of DNA? Although DNase is ubiquitous in nature, extracellular DNA has been found in many environments (i.e., wastewater, seawater, freshwater, sediments and soil) (Lorenz and Wackernagel 1994). The fate of extracellular DNA was monitored by examining the persistence and stability of a plasmid in three different soil types over a 60-day period (Romanowski et al. 1991). The plasmid remained in the form of circular and full-length linear molecules after one hour in the soil and the transforming activity of this plasmid was detectable up to 10 days after inoculation. The resistance of this DNA to nucleolytic degradation in these samples was attributed to the protection that minerals and other particle surfaces provide for the DNA. At first I assumed that the protection of DNA by MVs may not be as significant as I initially thought. But, even though naked DNA can maintain its transforming activity long enough to be transferred, this does not diminish the benefit of indiscriminate MV fusion to a Gram-negative bacterium (i.e., competent or noncompetent).

It seems that PAO1 MV-mediated genetic exchange does not occur under our laboratory conditions. Other variations to the transformation experiments can be applied to further investigate whether or not p-MVs do participate in transformation. The addition of an antibiotic (i.e., carbenicillin) to the transformation mixture may create a pressure that promotes the uptake of pAK1900 DNA by recipient cells. With no selective pressure the bacterium would not benefit in the uptake of this DNA. Lastly, these experiments can be done on a solid interface rather than in liquid. The probability of the interaction of MVs with recipient cells could be increased by concentrating a volume of

cells onto a filter. Thus, p-MVs could establish more frequent and stable contact which could possibly lead to transformation.

4.0 FURTHER DEFINING THE ORIGIN OF DNA WITHIN p-MVS

4.1 Background

Previous research has shown that MVs isolated from a number of bacterial species contain OMPs and are enriched with a variety of periplasmic components. Some examples are leukotoxin in MVs of *A. actinomycetemcomitans* (Kato et al. 2002), LT and alkaline phosphatase within MVs of ETEC (Wai et al. 1995; Horstman and Kuehn 2000), *P. aeruginosa* MVs containing protease, phospholipase C, PG hydrolase, alkaline phosphatase, and pro-elastase (Kadurugamuwa and Beveridge 1995; 1997), β-lactamase within MVs of β-lactam-resistant *P. aeruginosa* clinical isolates (Ciofu et al. 2000) and Shiga toxin 1 and 2 within the *E. coli* O157:H7 MVs (Kolling and Matthews 1999). Interestingly, this thesis work has demonstrated that plasmid DNA incorporates itself within MVs of PAO1/pAK1900. Unlike the periplasmic material mentioned above, DNA is not a permanent resident of the periplasm but instead exists within the cytoplasm. This then raises the question: how does DNA become internalized within periplasmic-containing MVs? Four possible models of this association have been formulated (Fig. 8). For three models the DNA is inserted into MVs before the vesicles leave the bacterium. Here the encompassing material could be PM (Fig. 8B), OM (Fig. 8C) or both (Fig. 8A). In the fourth model, the DNA would enter the MV after the vesicle has left the cell and the DNA would be exogenous (Fig. 8D).

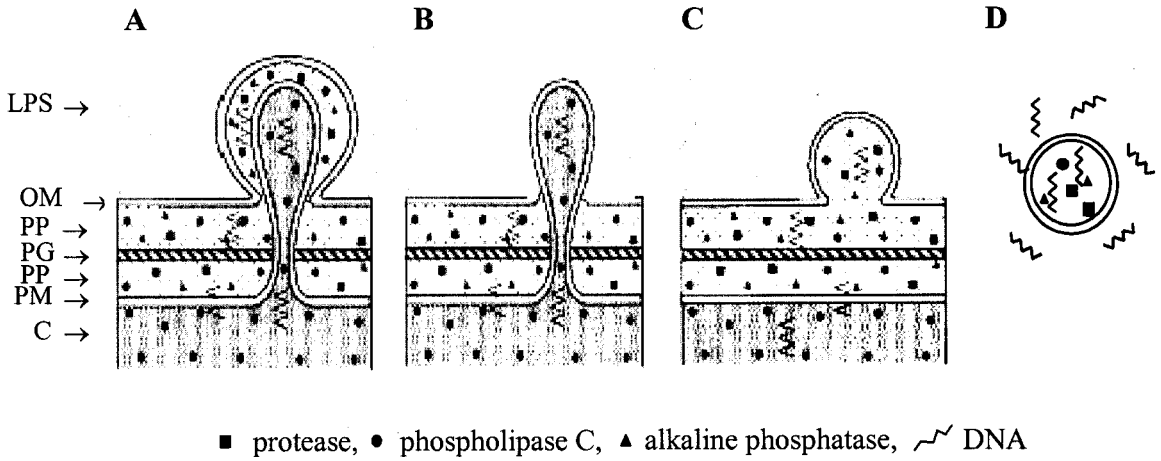

Fig. 8. Models representing DNA encapsulation within MVs. (A) OM and PM pinch-off forming a large double bilayered MV containing cytoplasm. (B) PM vesicle encompassing cytoplasm. (C) Migration of DNA from the cytoplasm into the periplasm, accompanying other periplasmic material that is being incorporated within an MV. (D) Free DNA in the environment is absorbed and internalized into an MV. PP, periplasm; C, cytoplasm. Adapted from Kadurugamuwa and Beveridge (1995).

4.1.1 Present Study

The objective of the experimentation in this present chapter was to help clarify the origin of DNA within PAO1/pAK1900 MVs. The four models of DNA encapsulation were examined by defining the structure and composition of MVs. This was attempted by a number of methods. The physical and ultrastructural differences between n- and p-MVs were compared by cryo-TEM. Isopycnic sucrose density centrifugation was done to determine the buoyant densities of MVs as compared to those of OM and PM. The protein and LPS profiles of MVs were examined by SDS-PAGE and enzyme assays specific to OM and PM were performed. Lastly, DNA encapsulation experiments were done to observe the MV-DNA association and were analyzed by PCR.

4.2 Materials and Methods

4.2.1 Isopycnic gradients and determination of buoyant densities (ρ)

OM and PM were purified as described previously (Hancock and Nikaido, 1978; Horstman and Kuehn, 2000) with some modification. Cells from an overnight 1 L culture of PAO1 and PAO1/pAK1900 grown at 37°C with shaking at 120 rpm were harvested by centrifugation (6,000 × g, 15 min). The cell pellet was washed twice in 100 mM HEPES (pH 6.8). The washed pellet was resuspended in 20 ml of cold 100 mM HEPES, 1 mg/ml lysozyme (Sigma), 50 μg/ml DNase type I, 100 μg/ml RNase type III-A (Sigma), 20% sucrose (wt/vol) and kept on ice. This suspension was passed twice through a cold French pressure cell (American Instrument Company) at 18,000 psi. The suspension was centrifuged (3,000 × g, 10 min) to remove any intact cells. The supernatant was layered on a sucrose cushion (0.5 ml of 5% and 2 ml of 55% sucrose (wt/wt) in 30 mM Tris (pH 8.0)) and centrifuged (183,000 × g, 3 h) in a SW 41 rotor (Beckman Instruments). 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 (0.5 ml of 60%, 0.9 ml of 55%, 2.2 ml of 50%, 2.2 ml of 45%, 2.2 ml of 40%, and 0.4 ml 35% sucrose (wt/wt) in Tris (pH 8.0)) and centrifuged to equilibrium (183,000 × g, 18 h) in a SW 41 rotor. Because sucrose is non-ionic and inert towards biological membranes (Rickwood 1978) it was used as the gradient media. The resulting bands were visualized by indirect light and carefully removed with sterile Pasteur pipettes. Each fraction was concentrated by centrifugation (200,000 × g, 30 min) and washed five times by centrifugation with sterile Nanopure deionized water. OM and PM were selected by the fractions that contained the highest Kdo and highest NADH

oxidase activity, respectively. p-MVs and n-MVs (twenty-five 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. To ensure that equilibrium of the gradient was achieved, samples throughout the sucrose gradient were removed before and after the 18 h centrifugation period and the refractive indices of these samples were measured.

4.2.2 EM

4.2.2.1 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; for n-MVs and p-MVs) or in deionized water (for OM vesicles) 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 lower than 1500 electrons/nm^2. Bilayer thickness measurements of the digital images were done using the analySIS software (Münster, Germany). Intact vs. lysed p-MVs and OM vesicles were counted.

4.2.2.2 Conventional TEM

Negative stains were prepared as described in Chapter 2. The morphologies of fractions n-MV1, n-MV2, p-MV1 and p-MV2 isolated by isopycnic gradients were compared.

4.2.3 Enzyme assays

4.2.3.1 Kdo

Kdo was determined by the thiobarbituric acid (TBA) method (Karkhanis et al. 1978) with purified Kdo (Sigma) as a standard. Samples of n-MV, p-MV and membrane fractions were treated with 0.04 M H_2SO_4 for 20 min at 100°C to release Kdo. Any insoluble material was removed by centrifugation (4,000 × g for 20 min). 0.5 ml of acid hydrolysate was mixed with 0.25 ml of periodic acid reagent (25 mmol periodic acid in 0.25 ml 62.5 mmol H_2SO_4) and incubated at 37°C for 30 min. Samples were cooled and 0.25 ml of sodium arsenite solution (2% (wt/vol) sodium arsenite in 0.5 M HCl) was added. To this, 0.25 ml of TBA (0.6% (wt/vol) adjusted to pH 9.0 with NaOH) was added and the mixture was heated at 100°C for 7.5 min. Before the sample was cooled, 1 ml of dimelthylsulfoxide (DMSO) (Sigma) was added. The sample was then measured against a reagent blank (no Kdo) at 548 nm. These readings were compared against a standard curve made with purified Kdo ranging from 0 to 20 μg/ml.

4.2.3.2 NADH oxidase

The measurement of NADH oxidase was followed by the method of Osborn et al. (1972). Incubation mixtures contained 50 mM Tris-HCl (pH 7.5), 0.12 mM β-NADH (Sigma), 0.2 mM dithiothreitol (Sigma), and the membrane fraction (50 to 100 μg of protein) in a volume of 1.0 ml. The disappearance of β-NADH was measured by the rate

of decrease in absorbance at 340 nm in a Shimatdzu UV-mini 1240 spectrophotometer (Mandel Scientific, Guelph, ON).

4.2.4 Polyacrylamide Gel Electrophoresis (PAGE)

Protein contents in samples were standardized based on protein determinations using the BCA protein assay. A 40 μg sample of protein from isolated membranes and vesicles was solubilized in sample buffer, (15.7% (vol/vol) Tris-HCl (pH 6.8), 3.1% SDS, 15.7% (vol/vol) glycerol, 3.1% (vol/vol) 2-mercaptoethanol, and 2.5% bromophenol blue) heated to 100°C for 10 min and analyzed. For LPS samples, 25 μl of proteinase K (solubilized in sample buffer to a concentration of 2.5 mg/ml) was added to the samples and was incubated at 60°C for 60 min. Samples were cooled and loaded onto polyacrylamide gels (5% stacking gel; 12% separating gel) and electrophoresed at 200 V. The gels were then stained with Coomassie Brilliant Blue R-250 (C.I. 42660) and destained with 5% (vol/vol) acetic acid (aq.). Silver staining was followed by the method of Tsai and Frasch (1982). Briefly, the gels were fixed for 45 min in a solution containing 40% (vol/vol) ethanol and 5% (vol/vol) acetic acid. The gels were then placed in an oxidizing solution of 0.75% (wt/vol) periodic acid in freshly prepared fixing solution for 30 min at room temperature. The oxidizing solution was removed by repeated washings in deionized water. Next, the gels were stained for 10 min in a solution of 0.67% (wt/vol) silver nitrate. This was followed by 10 washes in deionized water. Lastly, the gels were developed in BioRad silver stain developer (BioRad Laboratories Ltd., Mississauga, ON). Development was stopped by the addition of 2% (vol/vol) acetic acid and washed with deionized water.

4.2.5 DNA encapsulation assays

4.2.5.1 DNase assay

Two 500 ml TSB flasks were inoculated with PAO1/pAK1900. Flask A represented the control and Flask B was treated with a final concentration of 100 μg/ml DNase (Roche) and 10 mM $MgCl_2$. The flasks were incubated at 37°C (120 rpm) until late exponential phase and MVs were isolated. The internalization of DNA within MVs in the presence of DNase was determined by PCR (Chapter 2; Materials and Methods). 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 analyzed to determine whether extracellular DNA was present. Positive controls were purified pAK1900 and genomic whole-cell DNA. Heat inactivation of DNase (100°C for 15 min) was performed on samples before being analyzed by PCR (Tavares and Sellstedt 2001).

4.2.5.2 n-MVs + pAK1900

n-MVs (500 μg/ml) were incubated with 1 μg/ml of pAK1900 for 16 h at 37°C with slight agitation. Half of this sample was treated with DNase 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 of pAK1900 with 50 μg/ml DNase for 1 h at 37°C.

DNA encapsulated within n-MVs was quantified by the PicoGreen fluorometric DNA assay (Chapter 2: Materials and Methods).

4.3 Results

4.3.1 Isopycnic gradients

The buoyant densities of the membrane fractions and MVs were estimated using isopycnic sucrose density gradients (Table 3). An 18 h centrifugation period was applied to ensure that equilibrium of the gradient was reached (where the steps of sucrose are smoothed by diffusion) and thus a continuous gradient formed. This was verified by measuring the refractive index of samples throughout the gradient before and after centrifugation (Fig. 9).

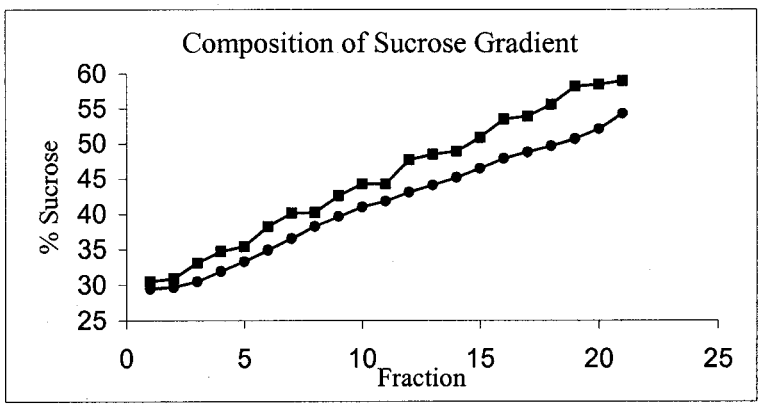

Fig. 9. Composition of isopycnic sucrose gradients before and after 18 h centrifugation. The refractive indices of sucrose fractions throughout the gradient were measured and converted to % sucrose by reference to the Index of Refraction Table: E-225 (Weast 1975). The (■) line represents the pre-centrifuged gradient and the (•) line represents the gradient after 18 h centrifugation.

A continuous gradient was achieved as seen by the smooth line of the 18 hr sample compared to the step-like line of the pre-centrifuged gradient. This gives confidence that the MVs and French-press whole cell lysates will migrate to the section of the gradient equal to its density. The fractionated membranes from whole cells of PAO1 and PAO1/pAK1900 banded at four discrete regions (Fig. 10), an observation consistent with

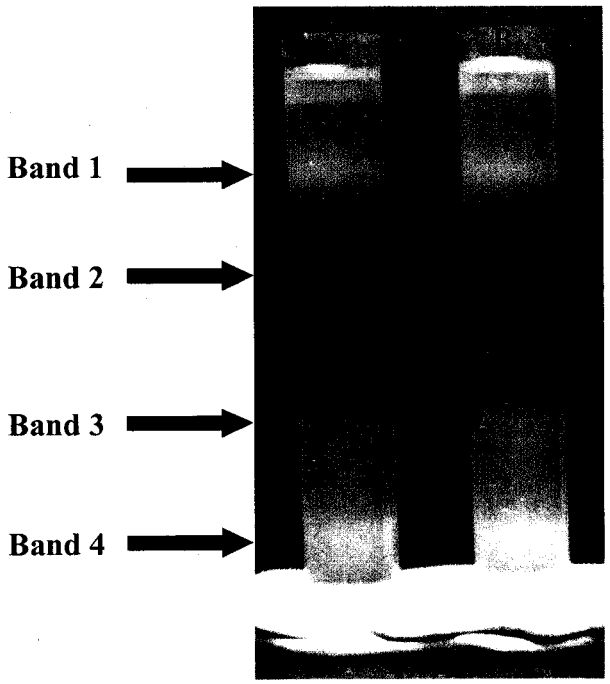

Fig. 10. Isopycnic density gradient centrifugation of membrane fractions prepared from PAO1 (Tube A) and PAO1/pAK1900 (Tube B). Arrows indicate the membrane banding points.

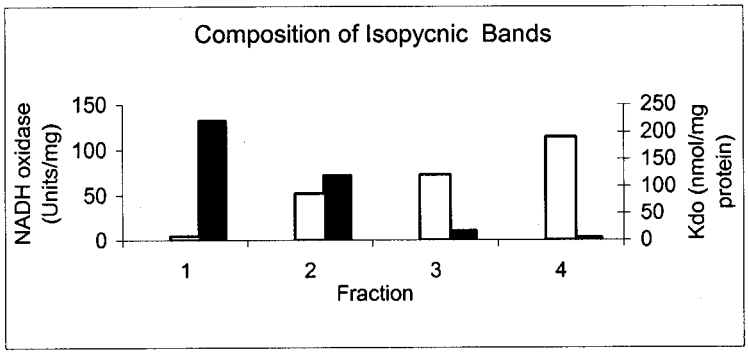

Fig. 11. Composition of isopycnic fractions from the bands seen in Tube A of Fig. 10. Fractions were assayed for Kdo and NADH oxidase activity. The white bar graph represents Kdo (nmol/mg protein) and the black bar graph represents NADH oxidase activity (Units/mg). Relative running positions are located on the x axis and are identified in Fig. 10. Kdo values and NADH oxidase activity are similar to those obtained by Mizuno and Kageyama (1978) and Hancock and Nikaido (1978), respectively.

Hancock and Nikaido (1978). In order to distinguish the origin of each of these bands, Kdo (OM marker) and NADH oxidase (PM marker) were measured (Fig. 11). Fraction 1 and 2 contained NADH oxidase activity with very little Kdo. Fraction 3 and 4 contained little NADH oxidase and high amounts of Kdo. These results when compared to observations by Hancock and Nikaido (1978) suggest that the PM is located in Fraction 1, PM plus OM in Fraction 2, and OM in Fractions 3 and 4. For comparison, the densities of purified PM and OM were determined (Table 3). The PM had a density of 1.170 g/ml and the two OM bands fractionated at densities 1.240 and 1.260 g/ml.

Both n- and p-MVs fractionated into two bands but at different densities (Fig. 12). p-MVs at ρ = 1.250 g/ml and 1.265 g/ml and n-MVs at the same density as the purified OM fractions. Each fraction was analyzed in the TEM to determine if there was any difference in MV size or morphology between the samples (Fig. 13). It is clearly shown that n-MV1, n-MV2, p-MV1 and p-MV2 all have a similar size range and are mainly spherical but with a small number of elongated and tubular vesicles present. A comparison of the protein profile (Fig. 14A) of these samples with OM indicate that the MVs from each fraction are identical to each other and similar to the OM. Although the LPS profiles (Fig. 14B) of the MV fractions are also similar to each other and to the OM, n-MV-2 (lane 3) is more intensely stained.

The difference in density between n-MV1 and n-MV2, and p-MV1 and p-MV2 could be linked to the presence of different periplasmic components in each vesicle fraction or by discrete regions of OM possessing distinctly different densities.

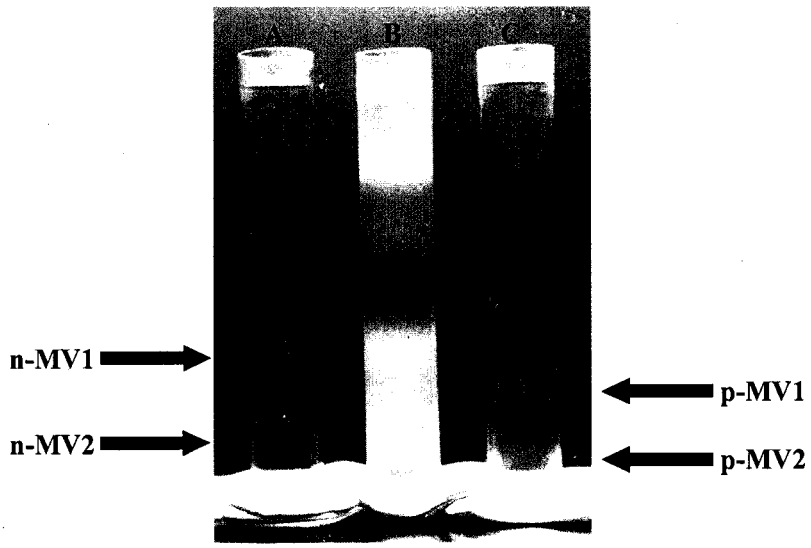

Fig. 12. Isopycnic density gradient centrifugation of n-MVs (Tube A), PAO1 membrane fractions (Tube B) and p-MVs (Tube C). Arrows indicate the MV banding regions.

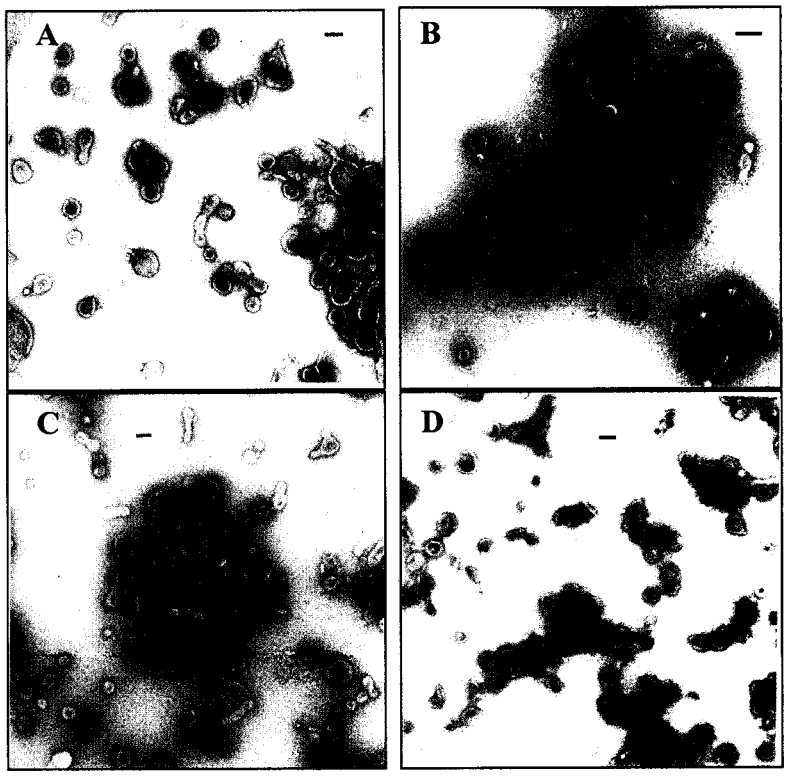

Fig. 13. Negatively stained whole mounts of isopycnic gradient MV fractions n-MV1 (A), n-MV2 (B), p-MV1 (C), p-MV2 (D) from the bands seen in Fig. 11. The scale bar represents 100 nm.

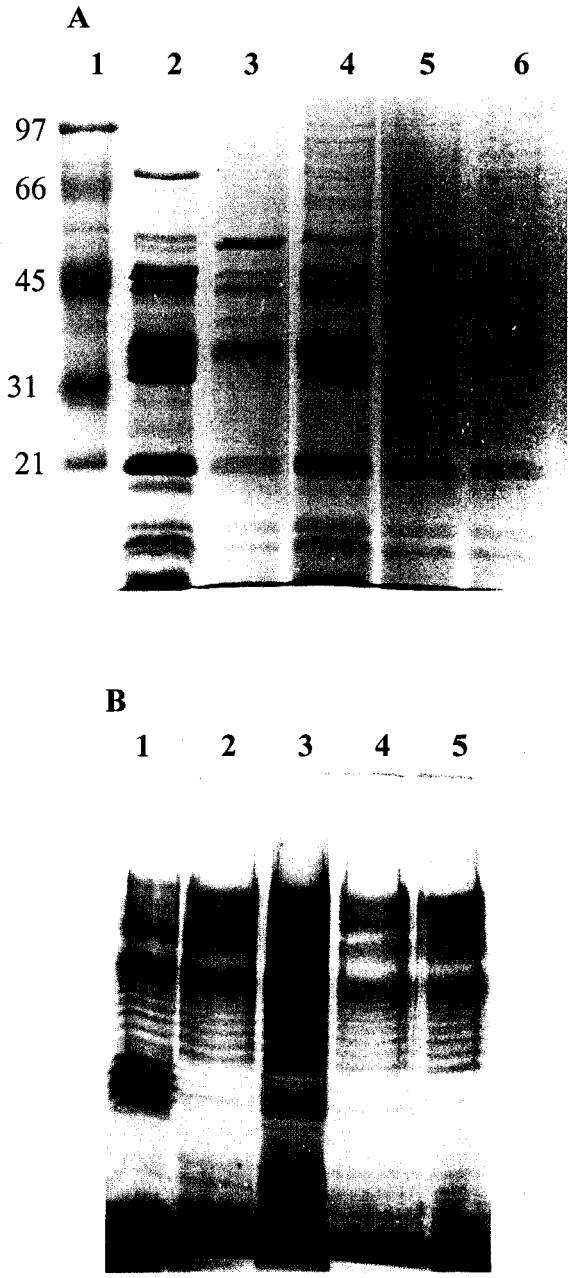

Fig. 14. Comparison of the protein and LPS profiles of MVs separated from isopycnic density gradients identified in Fig. 11. (A) SDS-PAGE protein profiles of PM, OM, n-MVs and p-MVs stained with Coomassie Brilliant Blue. Lane 1, commercially prepared molecular mass markers (kDa); lane 2, OM; lane 3, n-MV1; lane 4, n-MV2; lane 5, p-MV1; lane 6, p-MV2. (B) Silver-stained SDS-PAGE of LPS from PAO1. Lane 1, whole control cells; lane 2, n-MV1; lane 3, n-MV2; lane 4, p-MV1; lane 5, p-MV2.

4.3.2 Characterization and Composition of n- and p-MVs

The remaining experiments characterizing n- and p-MVs were done using MVs isolated by the original MV isolation procedure and not by applying to the sucrose density gradient.

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 3) and staining intensity to that of the PAO1 OM (Fig. 15).

When chemical 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 per mg of protein) with virtually no NADH oxidase (0.07 - 0.27 Units/mg) (Table 3).

The protein profiles of OM, PM, n- and p-MVs were compared by SDS-PAGE stained with Coomassie Brilliant Blue (Fig. 16A). 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 (i.e, OprC (70 kDa, esterase (55 kDa), OprF (38 kDa), and OprH2 (20.5 kDa)). While several bands seen in n-MVs and p-MVs correlated with OMPs from the OM fraction, there were some differences in stain intensity and distribution of band molecular weights. Bands 40 kDa and 65 kDa were present in MVs but were absent in OM. In addition, band 40 kDa was not found in the protein profiles of the MV fractions purified by isopycnic gradients (Fig 14A), suggesting that this protein may not be tightly associated with the MVs.

The carbohydrate moieties and banding patterns of OM, n- and p-MVs were analysed by silver stained SDS-PAGE (Fig. 16B). n- and p-MVs showed similar LPS banding patterns and demonstrated the characteristic ladder-like bands and core regions possessing similar relative mobilities to those of the whole cells. One noted difference in the LPS profiles was that the high molecular weight O-antigen region of n-MV profile was more predominant than p-MV and OM profiles.

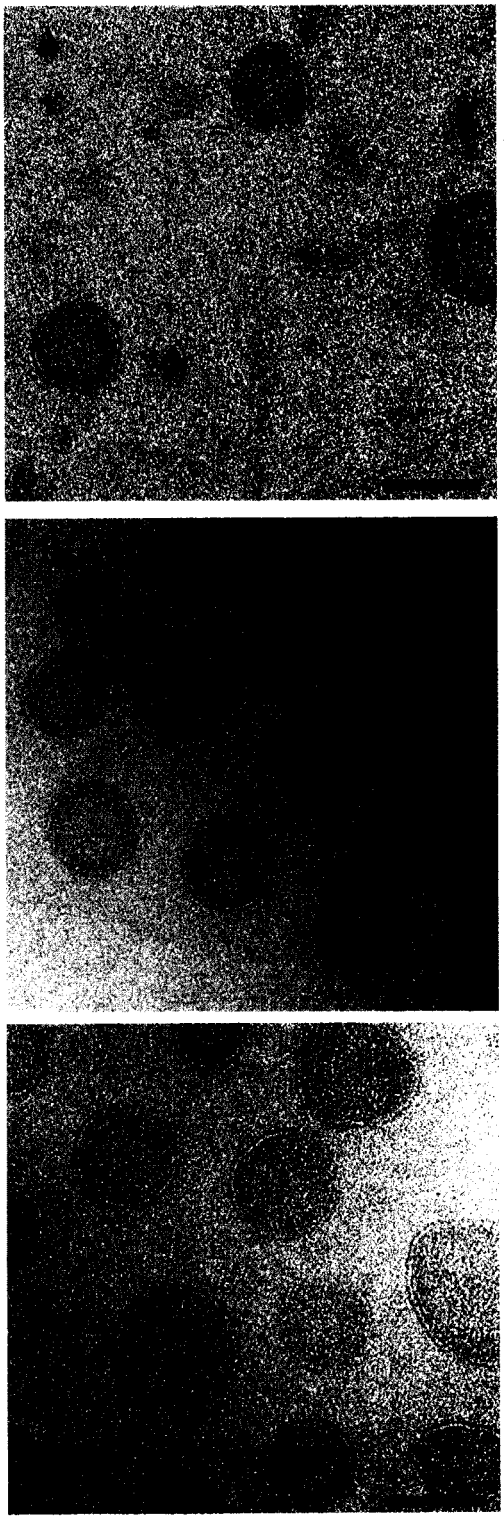

Fig. 15. Thin frozen films of n-MVs (A), p-MVs (B) and OM vesicles (C) observed by cryo-TEM. It is important to note that each vesicle type consists of a single membrane bilayer. Bar, 100 nm.

Table 3. 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)

	Thickness of bilayer (nm)[a]	Buoyant density (g/ml)	Kdo (nmol per mg of protein)[b]	NADH oxidase (Units/mg)[b, c]
PM	ND	1.170	7.72 ± 0.78	132.50 ± 20.43
OM	5.39 ± 0.49	1.240, 1.260	190.97 ± 47.15	2.99 ± 2.30
n-MVs	5.42 ± 0.62	1.240, 1.260	41.02 ± 6.24	0.27 ± 0.32
p-MVs	5.49 ± 0.52	1.250, 1.265	43.70 ± 6.02	0.07 ± 0.02

[a] $n = 20$ (avg ± SD)
[b] $n = 3$ (avg ± SD)
[c] One unit = nanomoles per min

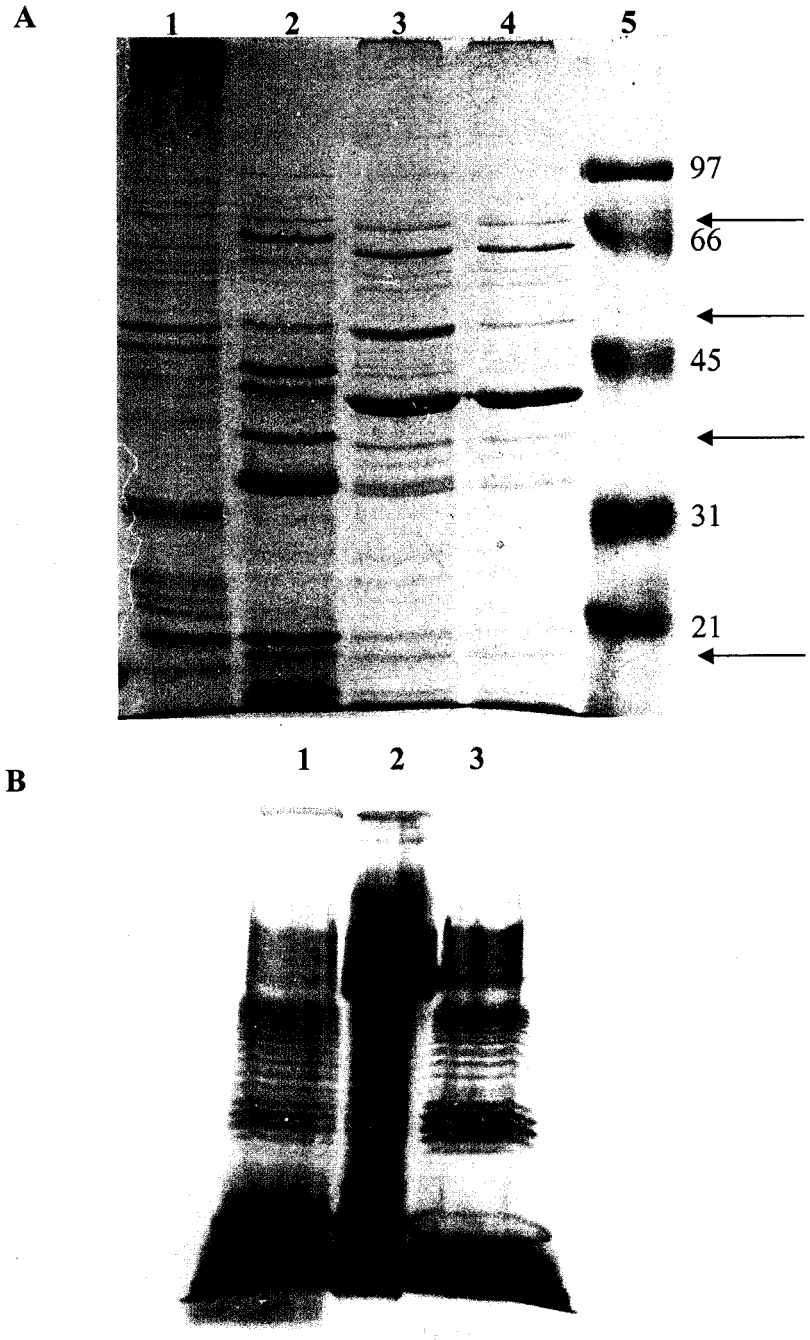

Fig. 16. (A) SDS-PAGE protein profiles of PM, OM, n-MVs and p-MVs stained with Coomassie Brilliant Blue. Lane 1, PM; lane 2, OM; lane 3, n-MVs; lane 4, p-MVs; lane 5, commercially prepared molecular mass markers (kDa). OMPs OprC (70 kDa), esterase (55 kDa), OprF (38 kDa), OprH2 (20.5 kDa), according to the electrophoretic classification of OMPs by Hancock et al. (1990) were identified in OM and MV fractions (arrows). (B) Silver-stained SDS-PAGE of LPS from PAO1 whole control cells (lane 1), n-MVs (lane 2) and p-MVs (lane 3).

4.3.3. Encapsulation of Exogenous DNA

It is important to note that the preparation of MVs used in this experimentation differs from that of Chapter 2, where p-MVs were first isolated from an overnight culture followed then by DNase treatment. Here, in this chapter, MVs of PAO1/pAK1900 were isolated from an overnight culture continuously grown in the presence of DNase. This study was performed to determine whether pAK1900 becomes encapsulated within MVs due to extracellular DNA present in the surrounding culture fluid or due to the presence of DNA in the periplasm. The encapsulation of pAK1900 within the MVs was determined by PCR (Fig. 17). The presence of extracellular chromosomal and plasmid DNA was detected in the ultracentrifuged supernatant of Flask A (containing no cells and MVs) (lane 5, 7). No DNA was present in the cell- and MV-free supernatant of Flask B (DNase-treated culture) (lane 6, 8). Chromosomal and plasmid DNA was associated with MVs isolated from Flask A (lanes 9, 11). Interestingly, MVs isolated from the 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.

A second assay was performed to examine whether MVs incorporate extracellular DNA. n-MVs incubated with purified pAK1900 for 16 h followed by DNase-treatment were assayed by PCR (Fig. 18). Complete hydrolysis of pAK1900 with DNase occurred (lane 6), while n-MVs + pAK1900 appeared unaffected by the enzyme (lane 5). The amount of DNA internalized within the n-MVs was determined by fluorometric analysis. By comparing the DNA concentrations of n-MVs incubated with (Table 4) and without pAK1900 (Chapter 2, Table 1), n-MVs + pAK1900 contained 1.14 ng more DNA. When both of these MV fractions were DNase treated, thus measuring only internal DNA, an

increase of 0.34 ng of DNA in n-MVs + pAK1900 to n-MVs (without pAK1900) was measured. These results clearly indicated that exogenous plasmid DNA was internalized. It was possible that broken MVs had bound this amount of DNA and, once their membranes had reannealed, were responsible for encapsulation.

For these reasons, the MV stability of p-MVs and OM vesicles were analysed by comparing the number of intact to broken vesicles (Fig. 19). 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.

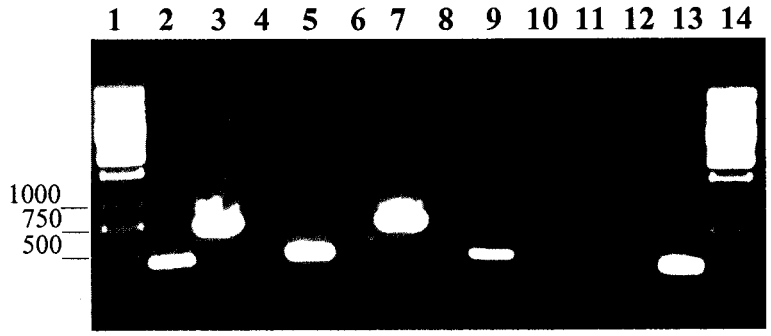

Fig. 17. PCR analysis of MVs isolated from DNase-treated PAO1/pAK1900 growth culture. Primers used are specific for *lac* (475 bp; lanes 2, 5, 6, 9, 10, 13) and for *oprL* (760 bp; lanes 3, 7, 8, 11, 12). Lane 1 and 14, 1 kb DNA ladder; lane 2, plasmid pAK1900 (control); lane 3, PAO1 genomic DNA (control); lane 4, no template (control); lane 5 and 7, ultracentrifuged supernatant of Flask A (no DNase); lane 6 and 8, ultracentrifuged supernatant of Flask B (DNase); lane 9 and 11, MVs isolated from Flask A; lane 10 and 12, MVs isolated from Flask B; lane 13, heat inactivated DNase (control). Molecular sizes in bp are as indicated on the left of each panel.

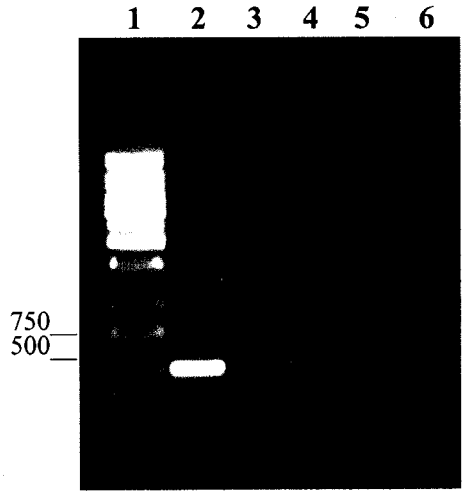

Fig. 18. PCR amplification of n-MVs following incubation with pAK1900. Primers are specific for *lac* (475 bp). Lane 1, 1 kb DNA ladder; lane 2, plasmid pAK1900 (control); lane 3, n-MVs alone; lane 4, n-MVs + pAK1900 (16 h incubation); lane 5, n-MVs + pAK1900 (16 h incubation) and DNase treated; lane 6, digestion of pAK1900 with DNase. Molecular sizes in bp are as indicated on the left of each panel.

Table 4. DNA content of n-MVs incubated for 16 h with pAK1900

Strain	Vesicle Type	ng of DNA/20 μg of MV protein (avg ± SD)[a]
PAO1	n-MV + pAK1900	1.45 ± 0.71
PAO1	n-MV + pAK1900 (DNase)	0.57 ± 0.11

MVs lysed with GES reagent.
[a] $n = 3$

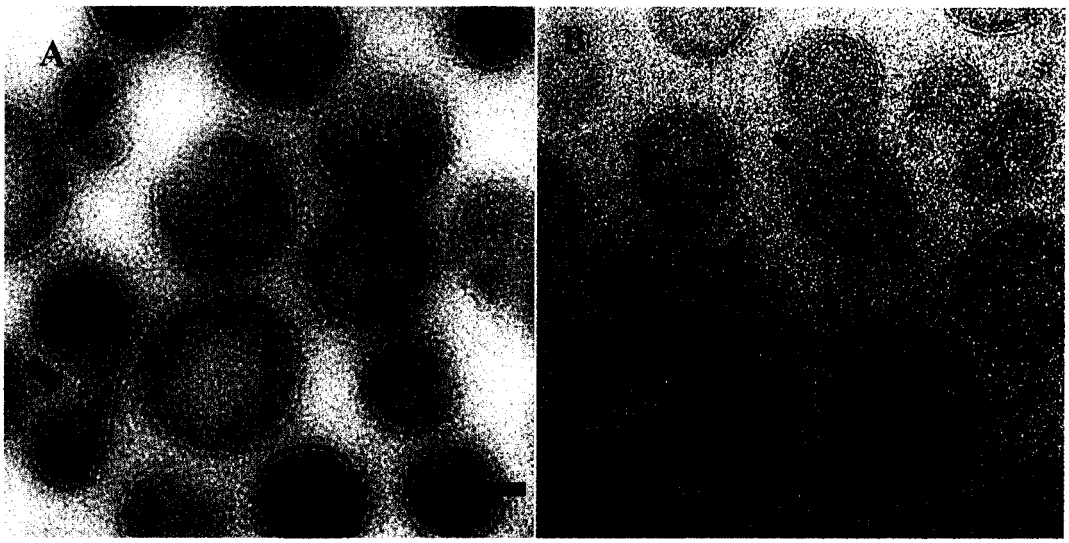

Fig. 19. Thin frozen films of (A) OM vesicles and (B) p-MVs observed by cryo-TEM. Arrows point to broken vesicle. Bar, 100 nm.

4.4 Discussion

MVs are routinely isolated from cells in the exponential growth phase and not in the stationary or death phase. Although it is unlikely that the production of DNA-MVs is due to cell lysis, it cannot be assumed that there are no lysed cells in the growth phase. To verify that MVs were not a product of cell lysis the ultrastructural and physical composition of MVs were examined. If MVs are lysis products, one would expect them to be either double-bilayered with an OM and PM, or only bound by the PM. Only single-bilayered MVs were observed by cryo-TEM and measurements of the thickness of this bilayer were almost identical to that of OM vesicles. Cryo-thin sections of *P. aeruginosa* whole cells viewed by cryo-TEM demonstrate that PM is thinner than OM (Matias et al. 2003). Since these preparations were snap-frozen and physically fixed in vitreous ice, they remain in a hydrated state and represent the most authentic and natural view of MVs.

To distinguish the makeup of this bilayer, i.e., whether it is OM- or PM-based, the buoyant densities of the MVs were compared using isopycnic sucrose density gradients. This technique allows for the separation of particles based on buoyant density; for example, if broken cells are applied to a sucrose gradient, during centrifugation the membranes will migrate to a point in the gradient where each fraction has the same density as the gradient solution. When the buoyant densities of n- and p-MVs were analyzed, each of the two MV types migrated to two different levels. n-MVs had a density of 1.24 and 1.26 g/ml, the same density as isolated OM. This observation is in accordance with results obtained for ETEC MVs where the MVs and OM migrated to identical positions in the gradient (Horstman and Kuehn 2000). In addition, Gankema et

al. (1980) found that MVs had a range of density of 1.20 - 1.28 g/ml, a value similar to the one obtained in this study. p-MVs were more dense than n-MVs (ρ = 1.25 and 1.265 g/ml) which may be attributed to the presence of DNA within p-MVs. DNA is a dense material (ρ = 1.68 g/ml) but when surrounded with a membrane composed of protein and lipid the overall density would less. An example of this is T2 phage which is composed mainly of protein (ρ = 1.25 - 1.35 g/ml) and DNA and these phage particles have a buoyant density of 1.55 g/ml (Sheeler and Bianchi, 1980). MVs are not only made of protein but also contain lipid which is less dense than protein. Knowing the densities of these components gives a good indication as to why p-MVs are more dense than n-MVs but less dense than naked DNA or T2 phage.

Protein and LPS profiles of p-MVs compared to OM gave further evidence that MVs are OM based. The protein patterns of n- and p-MVs resembled each other and contained most major OMPs but were not identical to the OM. This observation is consistent with other studies comparing MV and OM proteins (Deich and Hoyer 1982; Dorward and Garon 1989; Gankema et al. 1980; Grenier and Mayrand 1987; Horstman and Kuehn 2000; Kadurugamuwa and Beveridge 1995; Kahn et al. 1982; Kato et al. 2002; Kolling and Matthews 1999; Petit and Judd 1992 a and b; Zhou et al. 1998). This small difference could be attributed to the OMPs that are either not present or under represented in MVs. Also, proteins found only in MVs could exist in the OM but at very low levels. To further characterize which proteins are present in MVs Western immunoblots using antibodies specific to OMPs and sequencing those proteins that do not react to anti-OMPs should be performed.

Enzymatic 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 void of any PM.

Taken together, these results strongly suggest that p-MVs are OM bound and are not a product of cell lysis, thus disproving Models A and B of Fig. 8.

To investigate the two remaining models of DNA incorporation within MVs, DNA encapsulation experiments were performed. By growing cells in the presence of DNase, no extracellular DNA would exist in the supernatant and thus, would be accessible to MVs. Assuming that DNA is incorporated in MVs due to its possible presence in the periplasm, the addition of DNase would have no effect on the production of MVs containing DNA (p-MVs). Contrary to this, DNA was not found within MVs isolated by this method. To further examine this phenomenon, an additional assay was performed by incubating purified plasmid with n-MVs. When DNase treatment was performed, some intact DNA was detected subsequently by PCR, suggesting that this plasmid DNA was internalized and protected within the MVs. Fluorometric analysis of these MVs confirmed this finding. These results were unexpected and indicate that the incorporation of DNA within MVs does not mimic the mechanism by which periplasmic material is packaged (Model C). Instead, this evidence supports Model D where DNA existing freely in the surrounding environment manages to enter MVs via an unknown route.

Up to this point, only a few MV research groups have studied the interaction of DNA within MVs (Dorward and Garon, 1989; Dorward et al. 1989; Kadurugamuwa and Beveridge, 1995; Kolling and Matthews, 1999; Yaron et al. 2000). These former

experiments were limited to the detection of MV-DNA and the role that it may play in the genetic transformation of cells, but offer little information as to where this DNA originates and how it exists within an MV. Kahn et al. (1983) investigating transformasomes of *Haemophilus* indicated that a 29 kDa competence-specific protein present on these structures binds specific DNA and holds it tightly until it is packaged. They also indicated that the means by which this DNA enters the transformasome is unknown. Both this group and Deich and Hoyer (1982) suggested that DNA packaging within a transformasome may be analogous to viral DNA packaging. This method may occur in the *Haemophilus* system but may not apply to the *Pseudomonas* MV-DNA system. Unlike *Haemophilus* MVs that remain attached to the bacterial surface, *Pseudomonas* liberates MVs from its outer surface. For *Pseudomonas* MVs to transform other bacteria, DNA must be both contained within and delivered by the vesicles.

This work provides support for an alternate mechanism of MV-DNA encapsulation. If the structural integrity of some MVs is compromised, an influx of DNA within an MV could occur. It has previously been stated that once an MV is formed it exists as an energetically closed stable structure (Kadurugamuwa and Beveridge 1999). By examining thin frozen foils of p-MVs and OM vesicles by cryo-TEM a better understanding of their structural stability was achieved. Both p-MV and OM vesicle preparations contained approximately 7% broken vesicles. It is possible that once an MV breaks, DNA existing in the extracellular environment would enter. An MV would then be thermodynamically forced back into a low-energy intact vesicle (Kadurugamuwa and Beveridge 1999) enclosing to protect the DNA within.

From the observations obtained in this Chapter it seems clear that Model D represents the most likely method by which plasmid DNA becomes encapsulated within MVs. But, before completely eliminating Model C, it is important to revisit the results of Chapter 2. When p-MVs were DNase treated following MV isolation, they were found to contain internally associated pAK1900. Also, a larger amount of DNA contained in p-MVs was recovered (3.91 ng/20 μg MV protein). In this present study, n-MVs incubated with pAK1900 internalized approximately 15% of this amount of DNA (0.57 ng/20 μg MV protein).

It is difficult to reconcile these two results and values. Previous studies on environmental DNA concentrations suggest that my experimental exogenous DNA concentration of 1 μg/ml of pAK1900 DNA was either equal to or above that found in the environment (i.e., 1 μg/g has been found in a freshwater sediment sample (Ogram et al. 1987) and aquatic environments contain 0.2-44.0 μg/L (Lorenz and Wackernagel 1994)). One would assume that more DNA would be taken up by n-MV + pAK1900 if Model D is the only DNA encapsulation system. Yet, it is difficult to make this assumption considering the different methods of experimentation used in my two studies (cf., methods used in Chapter 2 and 4). First, the concentration of pAK1900 in the growing culture supernatant (Chapter 2) is unknown. Presumably, it may be equal to or more than the 1 μg/ml level of the Chapter 4 experiment. For this reason, if MVs are opening and closing during their isolation, some plasmid (and possibly chromosomal DNA) could be trapped. (No chromosomal DNA was found in the Chapter 2 experiments.) Another important difference of the two studies is that the earlier study uses an actively growing

culture and not purified MVs combined with DNA as in the later study, the latter representing a more artificial system.

If a small and variable proportion of p-MVs continually open and close as suggested above, a 16 h DNase treatment of PAO1/pAK1900 cells (Chapter 4) should make the DNA contained within susceptible to the enzyme; over time the total initial internal DNA concentration would be reduced thereby artificially negating the Model C mechanism. This possibility would explain the lack of PCR amplification of pAK1900 in p-MVs (Fig. 18). The p-MVs of Chapter 2 were DNase treated for only 1 h, a much shorter time frame so that not as much DNA would be hydrolyzed during the opening and closing of p-MVs. If this is correct, the DNA detected in p-MVs in Chapter 2 would contain a greater quantity of DNA.

At this point it is impossible to state unequivocally whether Model C, Model D or a combination of C and D is correct. More experimentation is required. Yet, I have attempted to provide a rational explanation for the divergence of DNA values found by the experiments of Chapter 2 and 4. Other explanations may be possible but, because of the discovery that a small proportion of MVs are open, I am biased towards a combination of Models C and D. I believe that an initial amount of plasmid DNA is derived from the periplasm and transported away from cells. Once en route, some MVs would break open, possibly liberating the encapsulated DNA. DNA that is still adhered to the inside of the open MV would be susceptible to close-by DNase. At the same time this open MV would be available to entrap additional DNA if it is in the vicinity, protecting them from DNase until they open again. This idea is just a scenario but, if

true, provides a more dynamic and complicated interaction for MVs and DNA than previously suspected.

It is clear that further studies are needed to provide a better mechanistic view of DNA-MV encapsulation. One such experiment would be to separate chromosomal DNA and plasmid DNA from the supernatant of growing PAO1/pAK1900 and measure the concentration of plasmid DNA. By comparing this value to that of the plasmid DNA added to n-MVs in Chapter 4, a better correlation could be made between the experiments of Chapter 2. Other experiments would be to measure the amount of DNA taken up by MVs isolated from the DNase-treated culture (empty p-MVs) and p-MVs (containing DNA). Also, by radiolabelling pAK1900 the fate of this plasmid could be tracked.

In conclusion the work presented here indicates that n-MVs and p-MVs are synonymous. If n-MVs are presented with the opportunity to exist in an environment containing plasmid DNA, they too can become p-MVs.

5.0 CONCLUSION

The main focus of this thesis was to characterize MVs from PAO1/pAK1900 with an emphasis on the MV-DNA and its potential role in genetic transformation. For the purpose of this study, it was crucial that all cells be eliminated from the MV preparations so that, in no way, would they contribute to the characterization of DNA-MVs. I was aware that during our laboratory's usual MV isolation procedure a small number of cells accompanied the MV preparation. These contaminating cells were identified as the MV-producing strain (i.e. PAO1, PAO1/pAK1900). The cells were eliminated by increased filtration and disinfection of the ultracentrifuge tubes. With this procedure in place I was assured that this work would be unaffected by contaminating cells. Quantification of DNA from MVs of PAO1/pAK1900 and PAO1 indicated that DNA was internally and externally associated with the MVs. Also, the amount of DNA within PAO1/pAK1900 MVs was greater than that of PAO1 MVs. Characterization of this DNA indicated that chromosomal DNA was only externally associated with both MV types but in the case of PAO1/pAK1900 MVs, plasmid DNA was found externally as well as internally, and thus the PAO1/pAK1900 MVs were termed p-MVs.

This finding sparked the idea that p-MVs may represent an adapted version of transformation, where encapsulated DNA would be protected by a membrane and this membrane would be structurally compatible with the OM of other Gram-negative bacteria (Fig. 20b). The benefits of these two properties are that DNA would not be susceptible to exogenous nucleases and that the fusion of a p-MV to a Gram-negative bacterium would be energetically favourable. Following MV attachment and with the assistance of the PG hydrolase also found within MVs, the genetic material would be

delivered into the periplasm of the recipient cell. Now, the DNA would only require transport across the PM where it would then have access to the cytoplasm. Unfortunately, using a variety of experimental conditions (i.e., various time points and media) transformation not observed. p-MVs incubated with PAO1 and *E. coli* lacking pAK1900 did not produce transformants. Because MVs have been previously shown to attach, fuse and deliver their components to the periplasm of other bacteria (Kadurugamuwa and Beveridge 1998; 1999), the lack of transformants could be a result of the inability of pAK1900 to cross the PM. Unlike competent bacteria, *P. aeruginosa* lacks competence specific proteins that allow for this transport (Dubnau and Provvedi 2000). Surprisingly, studies done by other researchers with *E. coli*, also noncompetent, have shown that transformation does occur via MVs (Kolling and Matthews 1999; Yaron et al. 2000). This could either indicate that competence is not required and, unlike *E. coli*, *P. aeruginosa* MVs lack the ability to transform or, as explained previously, the quality of the *E. coli* experimentation could have resulted in false transformants. Additional studies with *Neisseria* and *Haemophilus* have also observed this role for MVs in transformation (Dorward et al. 1989; Kahn et al. 1983). Further studies with other recipient strains and the use of different plasmids could provide more insight into whether *P. aeruginosa* MVs do provide this function.

If not involved in transformation, the association of DNA with MVs could provide some function. One such possibility is that DNA-containing MVs contribute to the biofilm biomass. The matrix that glues the biofilm together is composed of exopolysaccharides, proteins and DNA (Sutherland 2001). Although DNA is not perceived as the 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 externally with MVs, which may enhance biofilm production (Fig. 20C).

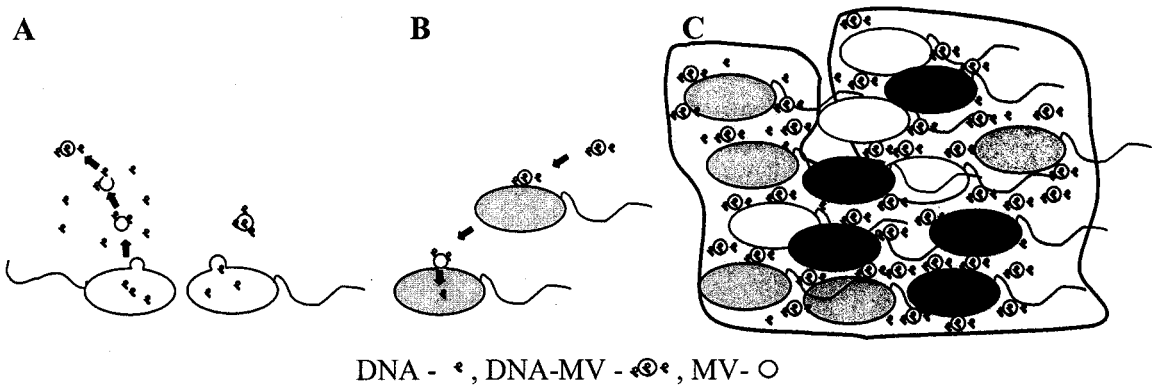

Fig. 20. Cartoon representation of formation of DNA-containing MVs (A), the potential MV-DNA may have in genetic transformation (B) and their contribution to the mass of a biofilm matrix (C).

The next aspect of this research was to determine the method by which DNA becomes internalized within MVs. Because MVs contain periplasmic components and DNA is found in the cytoplasm, its presence in MVs was perplexing. If MVs are products of lysis then DNA should be associated with fragments of PM, which would anneal to encapsulate the DNA. This work indicates that MVs are OM-based with no PM, and thus verifies that MVs are not products of lysis.

Two other methods of DNA encapsulation were explored (Fig. 20A); 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 attaches

to an MV and is somehow internalized. It is also possible that a combination of #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. 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 seems too high for this system to act alone. Although the results are inconclusive, most evidence points towards a combination of #1 and #2, and I believe the incorporation of DNA within MVs occurs by these two routes. To convincingly make such a statement, additional experiments are needed to clearly demonstrate this. For example, quantifying the amount of DNA within isolated p-MVs incubated with DNase over a range of time points (i.e., 1 – 16 h) could indicate whether the opening and closing of MVs results in the loss of encapsulated DNA over time.

Although other studies have looked at DNA-containing MVs (Dorward et al. 1989; Kadurugamuwa and Beveridge 1995; Kahn et al. 1983; Kolling and Matthews 1999; Yaron et al. 2000), these researchers did not thoroughly characterize their systems. Whole cell contamination of MV preparations was not accurately studied, (often) the MVs were not adequately chemically analyzed, and the MVs were not examined by cryo-TEM to help determine their physical properties. These previous studies were fraught with inconsistencies because the researchers were not thoroughly familiar with their respective MV systems. Indeed, even after approximately a decade of studying the PAO1 MV system, our own laboratory was not adequately appreciative of how important a small degree of whole cell contamination could be when it comes to a genetic process.

Though more work is needed to completely understand the association of DNA with MVs, this study goes beyond the work done by others in the MV-DNA field. Clearly, MVs encapsulate DNA and, with further evaluation, the complexity and importance of this internalization will be uncovered.

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