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Virulence Factors Are Released from *Pseudomonas aeruginosa* in Association with Membrane Vesicles during Normal Growth and Exposure to Gentamicin: a Novel Mechanism of Enzyme Secretion

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Pseudomonas aeruginosa **blebs-off membrane vesicles (MVs) into culture medium during normal growth. Release of these vesicles increased approximately threefold after exposure of the organism to four times the MIC of gentamicin. Natural and gentamicin-induced membrane vesicles (n-MVs and g-MVs, respectively) were isolated by filtration and differential centrifugation, and several of their biological activities were characterized. Electron microscopy of both n-MVs and g-MVs revealed that they were spherical bilayer MVs with a diameter of 50 to 150 nm. Immunoelectron microscopy and Western blot (immunoblot) analysis of the vesicles demonstrated the presence of B-band lipopolysaccharide (LPS), with a slightly higher proportion of B-band LPS in g-MVs than in n-MVs. A-band LPS was occasionally detected in g-MVs but not in n-MVs. In addition to LPS, several enzymes, such as phospholipase C, protease, hemolysin, and alkaline phosphatase, which are known to contribute to the pathogenicity of *Pseudomonas* infections were found to be present in both vesicle types. Both types of vesicles contained DNA, with a significantly higher content in g-MVs. These vesicles could thus play an important role in genetic transformation and disease by serving as a transport vehicle for DNA and virulence factors and are presumably involved in septic shock.**

Adverse effects on patients undergoing therapy resulting from antibiotic-induced liberation of bacterial components have been a long-standing concern (20, 39). Several different antibiotics can deleteriously affect the bacterial surface, especially by liberating endotoxin from gram-negative bacteria (20, 36, 37, 39, 52) and cell wall components of gram-positive bacteria (45). These components appear to be powerful inflammation inducers during infection, e.g., bacterial meningitis (24, 36, 45, 52).

The bactericidal activity of the aminoglycoside antibiotic gentamicin on *Pseudomonas aeruginosa* is partially mediated through perturbation of the cell surface (23, 25); the outer membrane becomes loosely attached to the bacterium at specific sites and forms membrane vesicles (MVs) which are cast from the cell to reside free in the external milieu. Many virulence factors of *P. aeruginosa* are cell surface components, such as lipopolysaccharide (LPS) and alginate, or extracellular products, such as alkaline protease, elastase, exotoxin A, DNase, and phospholipase C (PLC); in concert or acting alone, they contribute to the pathogenesis of infection (1, 2, 13, 14, 18, 37, 48, 54). Several studies have demonstrated the secretion of these products to the external environment during the growth of the pathogen (2, 3, 18, 19, 21, 28, 30).

Considerable effort has been devoted to understanding the mechanism of protein translocation in gram-negative bacteria (30, 32, 40). However, the precise events leading to the actual liberation of protein from the bacterial surface to the outside environment are not yet fully understood. A number of distinct modes of protein secretion exist (32, 40, 50), and in *P. aeruginosa*, at least two different secretion pathways have been identified (11, 13, 16, 29, 30, 50). Alkaline protease, a product of the *apr* gene, is believed to cross both inner and outer membranes in a single step (the signal peptide-independent pathway), possibly through membrane adhesion sites (or Bayer's junctions), thereby bypassing the periplasm, and this pathway is similar to the α-hemolysin pathway in *Escherichia coli* (13, 30, 40, 50). Although this model has a number of attractive features, the existence of such contact sites has been challenged (27). Most exoproteins in *Pseudomonas* spp. are believed to be secreted via a two-step mechanism. First, the proteins pass across the inner membrane in a signal peptide-dependent pathway (termed *sec*-dependent or general export pathway) with a transient stopover in the periplasm. The subsequent translocation across the outer membrane is mediated by specific helper proteins (encoded by *xcp* genes) composing a secretion machinery very similar to the well-characterized pullulanase system in *Klebsiella oxytoca* (40, 50). However, protein complexes spanning the periplasm and the outer membrane have not been demonstrated experimentally, nor has it been documented how they actually exert their function. (For example, the source of energy for the transfer of the exoproteins across the outer membrane is not yet clearly defined, despite evidence that a proton gradient is required for protein secretion in *P. aeruginosa* and *Aeromonas salmonicida* [46, 53].) Once the exoproteins are translocated across the plasma membrane into the periplasm, some folding of the proteins must occur, complete with their intramolecular bonding. Even tertiary or quaternary structures may result before the proteins are translocated across the outer membrane. How much complex, highly ordered structure can be tolerated by outer membrane protein (OMP) translocation systems? Presumably not much, since it would make the proteins too bulky for translo-

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cation. Therefore, these en route exoproteins in the periplasm may have to be unfolded before transport across the final membrane. It also possible that some exoproteins do not unfold but are actually released as packages in MVs.

This report demonstrates the release of MVs by *P. aeruginosa* during normal growth in culture. It is possible that these vesicles may function as a mechanism by which *P. aeruginosa* secretes natural products, such as virulence-associated factors, to the external environment. During infection, these MVs would concentrate these factors and convey them to the tissue which is to be infected. The natural release of MVs was increased severalfold on exposure of the organism to the membrane-perturbing antibiotic gentamicin. We present our findings on the composition of purified naturally evolved and gentamicin-induced MVs (n-MVs and g-MVs, respectively). On the basis of these data, a model for the excretion of several extracellular enzymes in *P. aeruginosa* is presented.

(Parts of this study were presented at the 94th General Meeting of the American Society for Microbiology [22].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* H103 and ATCC 19660 were grown in Mueller-Hinton broth to the early stationary growth phase (ca. 10^8 CFU/ml) on an orbital shaker at 37°C with an agitation rate of 125 rpm. To support the production of PLC, 0.2% (wt/vol) choline (the [2-hydroxy methyl]-trimethyl ammonium chloride salt; Sigma Chemical Co., St. Louis, Mo.) was added to culture medium (42). Strain ATCC 19660 was used to detect elastase and protease activities, as the amounts produced by this strain were found to be larger than those produced by H103 under the culture conditions used in the study.

Antibiotic susceptibility test. The MICs of gentamicin were determined by dilution in Mueller-Hinton broth. The MICs for strains H103 and ATCC 19660 were 2 and 2.5 μg/ml, respectively.

Isolation and quantification of MVs. One liter of bacterial culture in early stationary growth phase was divided into two equal parts. To one part, gentamicin at a final concentration of four times the MIC was added, whereas the other served as a control. Both cultures were incubated for 30 min on an orbital shaker at room temperature. Cells were removed from the suspension by centrifugation at 6,000 × *g*. The supernatants were filtered sequentially through 0.45- and 0.22-μm-pore-size cellulose acetate membranes (MSI, Westboro, Mass.) to remove residual cells. MVs were recovered from the resulting filtrates by centrifugation at 150,000 × *g* for 3 h at 5°C in a 45 Ti rotor (Beckman Instruments, Inc., Toronto, Canada), and the vesicle mass was measured after the supernatant was carefully aspirated from preweighed ultracentrifuge tubes. The vesicle pellet was washed once with 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (pH 6.8) (Research Organics, Inc., Cleveland, Ohio), pelleted by centrifugation at 120,000 × *g* for 30 min, and resuspended in 50 mM HEPES buffer (pH 6.8) containing 0.5 mM dithiothreitol (Sigma) as a protective agent for —SH groups. For some experiments, MVs were resuspended in HEPES buffer without dithiothreitol.

Electrophoresis. OMPs were prepared with sodium *N*-lauroyl sarcosinate (Sarkosyl; Sigma) as described previously (8). Washed whole cells and MVs were solubilized in sample buffer (0.5 M Tris hydrochloride [pH 6.8], 3% sodium dodecyl sulfate [SDS], 20% glycerol, 1% 2-mercaptoethanol, and 0.002% bromophenol blue) and heated to 100°C for 10 min. A 25-μg sample of protein was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 12% acrylamide gels, and the polypeptides were stained with Coomassie blue as described previously (8). A 40-μg sample of protein from whole cells and MVs was digested with proteinase K (100 μg/ml) (Sigma) and analyzed for LPS as described previously (25, 31).

Zymography. Proteinases were resolved by SDS-PAGE according to the method of Matsumoto et al. (34), with slight modifications. The separating gels used were 8% acrylamide gels containing α-casein or gelatin (type A from bovine skin; Sigma) at a final concentration of 0.15%. The stacking gels consisted of 4% acrylamide gels without gelatin or casein. A 25-μg protein sample from each preparation (without reducing agents) was loaded onto gels and was run at 4°C for 90 min at 120 V. After electrophoresis, the gels were shaken at room temperature in a solution of 2.5% Triton X-100 for 45 min to remove the SDS. Subsequently, the gels were incubated at 37°C in incubation buffer (50 mM Tris-HCl [pH 8.0], 5 mM $CaCl_2$) with 5 mM EDTA for 18 h. The positions of the proteinases were identified after the gels were stained (0.5% Coomassie brilliant blue R-250, 10% acetic acid, 40% methanol) for 2 h and clear bands were identified.

Western immunoblotting. LPS samples were transferred from the SDS-PAGE to nitrocellulose and reacted with monoclonal antibodies (MAbs) specific for B-band serotype O5 or A-band polysaccharide as described previously (25, 31). Alkaline phosphatase was detected by the method of Tan and Worobec (44). Elastase and alkaline protease were detected with mouse polyclonal antisera to the purified enzymes which were kindly supplied by R. Birk, Wayne State University. Purified *P. aeruginosa* elastase was from Nagase Biochemicals, Tokyo, Japan.

Fluorometric quantification of DNA. The DNA in MVs was quantitated by an assay developed by the Pierce Chemical Co. (Rockford, Ill.) according to the manufacturer's instructions. Briefly, 20 μg of protein from MVs in 50 μl of assay buffer (0.1 M NaCl, 10 mM EDTA, 10 mM Tris; pH 7.0) was lysed with 50 μl of extraction solution (0.1 M NH_4OH, 0.2% Triton X-100). A standard curve for DNA was prepared with calf thymus DNA (0 to 150 ng/ml) (provided with the assay kit) in 200 mM NaCl–20 mM EDTA (pH 7.0)–0.05 NH_4OH–0.01% Triton X-100. To each sample, 1.5 ml of fluorescent dye (200 μg/ml) (bisbenzimidazole) was added, the tubes were capped quickly and mixed, and fluorescence was measured in a Hitachi F-2000 fluorescence spectrophotometer with excitation and emission wavelengths set at 350 and 455 nm, respectively (10-nm slit width), yielding values for total DNA per milligram of protein. Experiments were also performed on MVs without their being treated with extraction solution. For some experiments, the intact MVs and purified DNA were treated with pancreatic DNase I (1.0 μg/ml; Sigma).

Preparation of cell lysates, supernatants, and MVs for enzyme assays. Membrane-filtered supernatants, before and after harvesting of MVs, were concentrated 10-fold in a Concentrator evaporator (Jouan, Winchester, Va.). Washed whole cells or MVs were sonicated for 2 min with 0.1% (vol/vol) toluene to release intracellular enzymes in a sonic bath (Bransonic Ultrasonic Corporation, Danbury, Conn.). Protein concentrations of samples were determined with the micro bicinchoninic acid reagent kit (Pierce). Whole cells and MVs (both at a 20-μg protein concentration) or concentrated supernatants (50 μl) were assayed for enzyme activity.

Enzyme assays. PLC activity was determined by using the synthetic substrate *p*-nitrophenyl phosphorylcholine (NPPC) (Sigma), as described by Berka et al. (3). Protease was determined by the sensitive assay described by Howe and Iglewski (19) using Hide powder azure (Sigma), and alkaline phosphatase was assayed by using *p*-nitrophenyl phosphate (pNPP) (Sigma), as previously described (44). Elastase activity was estimated by using elastin Congo red (Sigma) as a substrate in an assay based on the method of Kessler and Safrin (28). Hemolysin activity was measured as described by Bergmann et al. (1). Each sample was assayed with three replicates. The means and standard errors were calculated from at least three separate experiments.

Transmission electron microscopy (TEM). (i) Negative stains. A 20-μl volume of purified MVs was placed on carbon- and Formvar-coated nickel grids, which were then stained with 2% aqueous uranyl acetate, rinsed, and examined with a Philips EM300 transmission electron microscope operating under standard conditions at 60 kV with the cold trap in place.

(ii) Immunolabelling of thin sections. *P. aeruginosa* cells or purified MVs were enrobed in 2% molten Noble agar, put through a mild fixation-LR (London Resins) white embedding regimen, and indirectly labelled with either protein A-gold or anti-mouse immunoglobulin M-gold (EY Laboratories, San Mateo, Calif.) (all as outlined by Beveridge et al. [6]) using polyclonal antibodies or MAbs.

RESULTS

TEM. Figure 1 shows thin sections of *P. aeruginosa* cells which were either treated with gentamicin or untreated. The untreated control cells (Fig. 1A) represent natural cultured cells and possessed intact cell envelopes, with several membrane blebs emanating from each cell surface or free in the environment. Cells that were exposed to gentamicin formed many more blebs (Fig. 1B) than untreated cells. At a gentamicin concentration of 8 μg/ml, this increase in blebbing was visible after approximately 1 min of antibiotic incubation. Examination of intact isolated purified blebs from both natural and gentamicin-treated cultures in negative stains showed that although they were partially collapsed, many were filled with a particulate substance (Fig. 2A shows g-MVs). This was better shown and confirmed with thin sections (Fig. 2B). The diameters of the vesicles from both untreated and gentamicin-treated cells varied between 50 and 150 nm when measured in thin sections; however, when measurements of g-MVs were averaged, the g-MVs were found to be slightly larger than n-MVs, with a mean diameter of 100 nm as opposed to 80 nm for n-MVs. Thin sections proved the vesicles to have a bilayer structure (Fig. 2B). No external material was seen by any TEM technique, thereby suggesting that the isolated vesicles were free from particulate cellular debris.

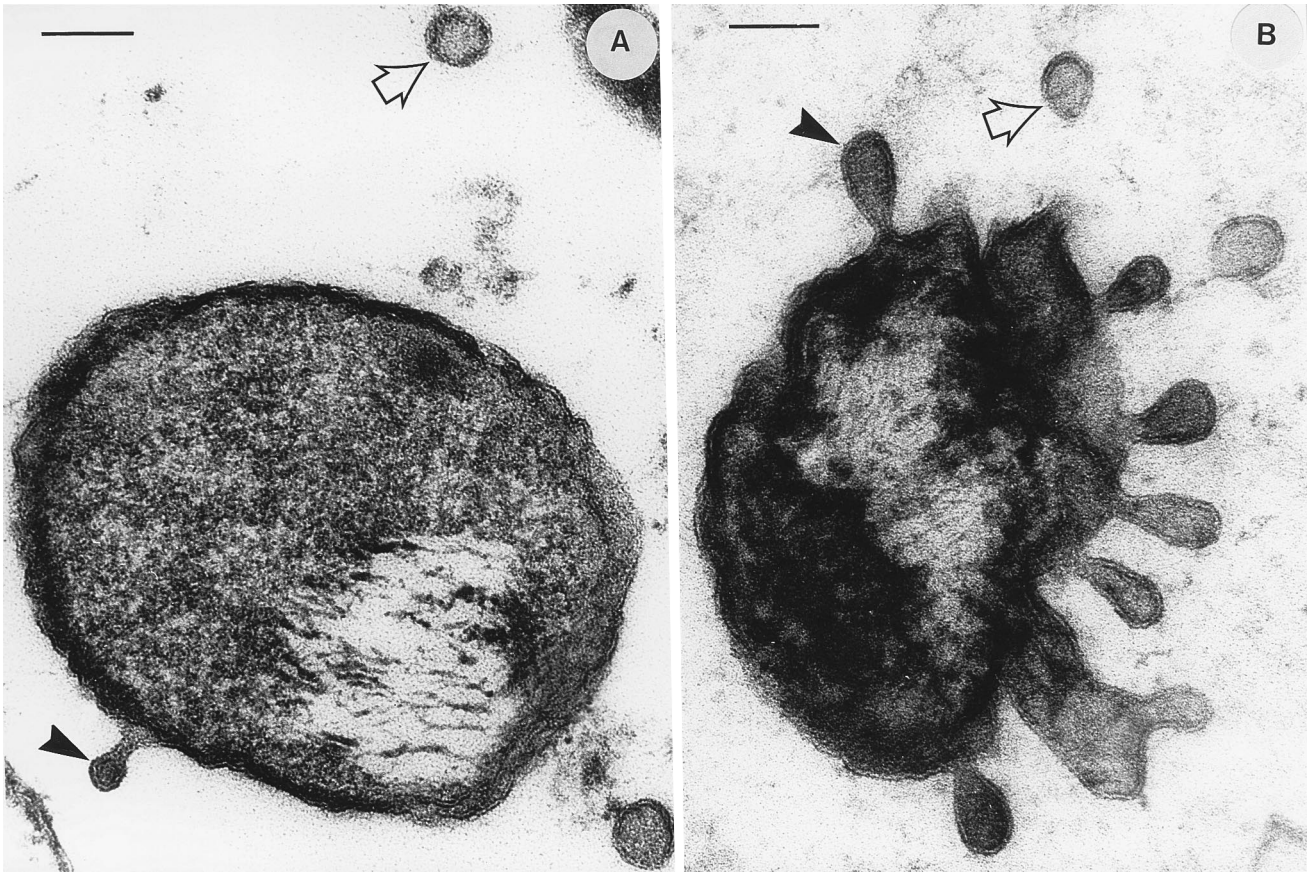

FIG. 1. Thin sections of H103 cells showing the formation of vesicles (solid arrowheads) and free MVs in growth medium (open arrows). (A) Control; (B) cells exposed to four times the MIC of gentamicin. Note that a larger number of MVs are formed from the cell surface of bacteria exposed to gentamicin than from untreated cells. Also note that electron-dense material has been trapped in the developing and free vesicles. Bars = 100 nm.

Mass differences between total n-MVs and g-MVs. A threefold increase in vesicle mass from bacteria exposed to gentamicin (36.5 ± 2.6 μg of vesicles [mean ± standard deviation] per mg of bacteria) in comparison with the untreated control cells (11.8 ± 1.8 μg of vesicles per mg of bacteria) was seen. This indicated that the antibiotic caused the release of more vesicles, presumably because of an ionic interaction between the antibiotic and the cell envelope, and confirmed the outer membrane destabilization demonstrated previously (25, 33). This increased mass of vesicles was in agreement with their increased frequency as seen by TEM (cf. Fig. 1A and B).

SDS-PAGE analysis of MVs. The protein profiles of whole-cell lysates, OMPs extracted from whole cells, and MVs from untreated or treated cells were compared by SDS-PAGE (Fig. 3). The n-MVs and g-MVs contained much fewer protein bands than the OMPs extracted from whole cells or whole-cell lysates. The banding patterns of n-MVs and g-MVs were very similar but not identical; both types of MVs appeared to have lost several bands which were normally present in whole-cell lysates and the OMP samples. Some of the prominently stained bands from both vesicle preparations included ~70-, 40-, and 20-kDa proteins. Trace amounts of an ~35-kDa protein was detected in g-MVs but not in n-MVs.

SDS-PAGE banding pattern and immunoreactivities of LPSs from MVs. Most *P. aeruginosa* strains coexpress two chemically and immunologically distinct types of LPS when grown in laboratory culture, namely, A-band and B-band LPSs (31, 41). LPS samples were separated by SDS-PAGE and silver stained to characterize the carbohydrate moieties and banding patterns (Fig. 4A). To identify the A- and B-band LPSs, electrophoretic blots of LPS from the SDS-PAGE whose results are shown in Fig. 4A were reacted with either B-band (Fig. 4B) or A-band-specific MAb (Fig. 4C). The ladder-like banding patterns of LPSs from both control and gentamicin-treated cells were similar to the patterns reported previously (25, 31) and showed similar irregularities in the spacing and intensities of the bands. In addition, both types of MVs showed the presence of ladder-like LPS bands and core regions with relative mobilities similar to that of whole cells (Fig. 4A). However, the ladder-like banding pattern and intensity of the core region appeared to be less extensive in n-MVs (Fig. 4A). As expected, immunoblotting of gentamicin-treated or control cell LPS reacted with both A-band- and B-band-specific MAbs showed coexpression of the A and B bands, confirming the results of earlier studies (25, 31). In contrast, Western blots of LPSs from n-MVs and g-MVs reacted somewhat differently with B-band-specific MAb. g-MVs possessed an increased amount of B-band LPS, and its banding pattern was similar to that of whole-cell extracts (Fig. 4B). The reaction of LPS from g-MVs with A-band-specific MAb was weakly positive, while n-MVs did not demonstrate the presence of any A-band LPS whatsoever (Fig. 4C). This confirms that gentamicin has a greater affinity for highly charged B-band LPS, so much so that B-band LPS is released in greater quantities to enrich g-MVs

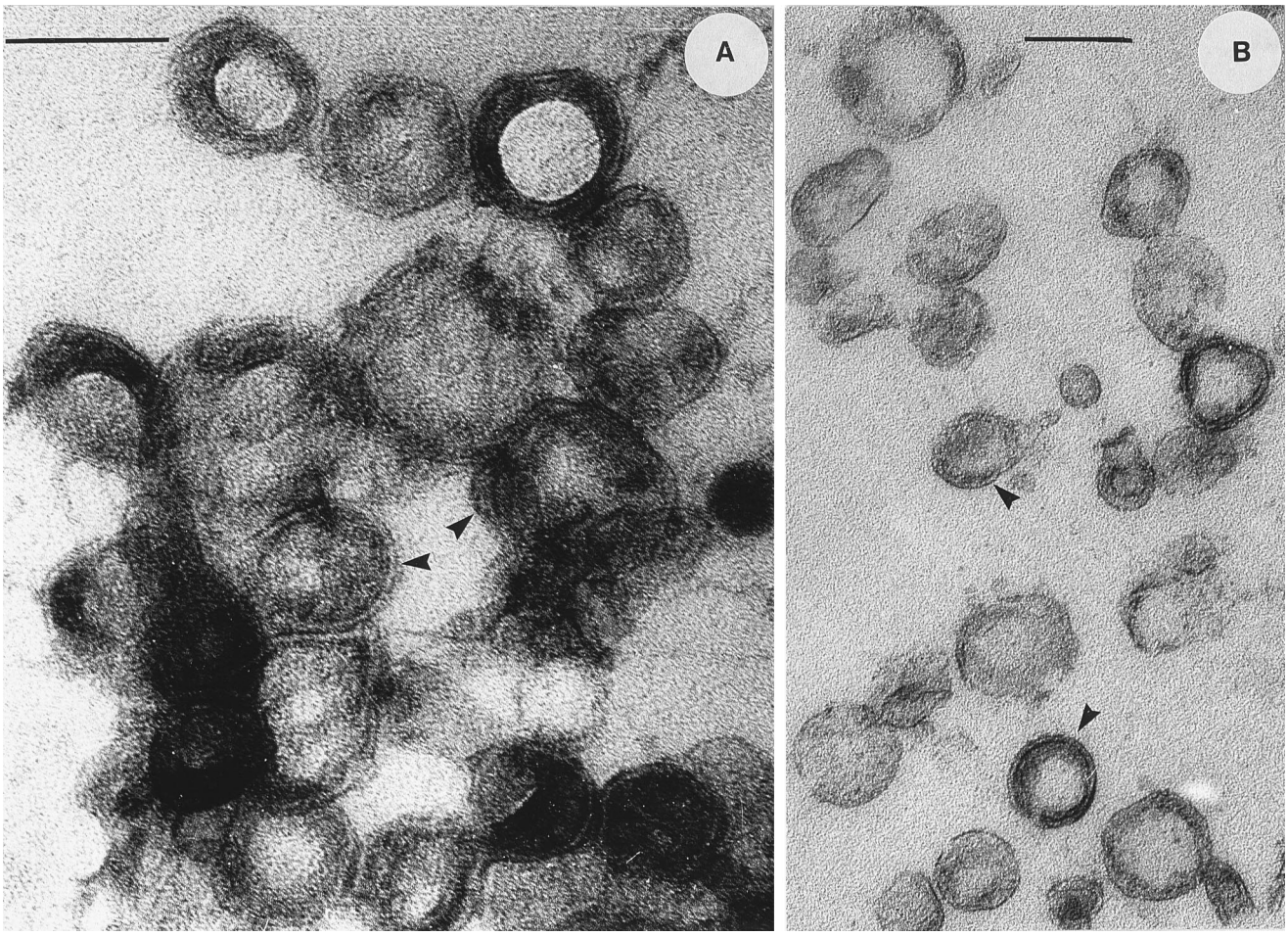

FIG. 2. Electron micrographs showing a negative stain (A) and a thin section (B) of intact g-MVs. Note that the spherical MVs are of various sizes (50 to 150 nm), possess an intact bilayer, and enclose electron-dense material (arrowheads). Bars = 100 nm.

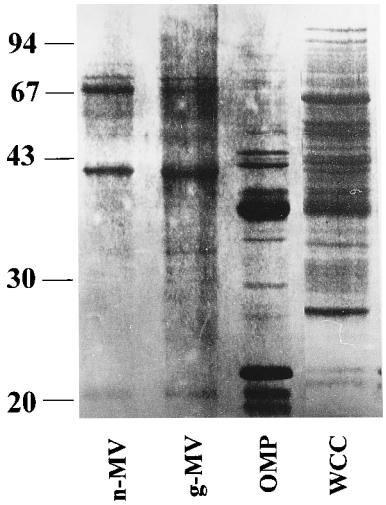

FIG. 3. SDS-PAGE protein profiles of n-MVs, g-MVs, OMPs, and whole control cells (WCC) in a 12% polyacrylamide gel stained with Coomassie brilliant blue. Each lane contains 25 μg of total protein from the indicated samples. Molecular masses (in kilodaltons) are indicated on the left.

(25). These results were further confirmed by immunogold electron microscopic detection of LPS on thin sections of n-MVs (Fig. 5A) and g-MVs (Fig. 5B).

Enzyme activities. Tables 1 and 2 illustrate the enzymatic activities in cellular extracts, MVs, and culture supernatants from cultures which were treated with gentamicin or untreated. Both types of vesicles exhibit PLC activity, as measured spectrophotometrically by the hydrolysis of NPPC, indicating that the enzyme is associated with the MVs. To evaluate the PLC activity in the supernatants, the enzyme activity was assayed before and after the removal of vesicles from cell-free culture supernatants. Removal of vesicles from gentamicin-treated cultures resulted in an 83% reduction in enzyme activity, compared with a 68% decrease in untreated cultures (Table 2). This suggests that the majority of PLC secreted into the external environment is indeed concentrated in the vesicles. The observed difference in enzyme activity between the two cultures is due to the fact that the amount of vesicles per unit mass is greater in gentamicin-exposed cultures than in untreated cultures, hence a higher percentage of PLC activity is removed with the vesicles. It has been reported previously that *P. aeruginosa* produces and excretes two distinct PLCs with similar activities; each is capable of acting on the substrate phosphatidylcholine (42). Although both PLCs hydrolyze this substrate, one is hemolytic (PLC-H) for sheep and human erythrocytes and is heat labile, while the other (PLC-N) is not.

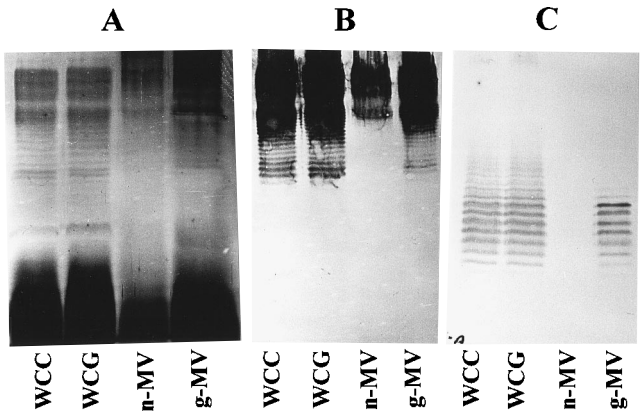

FIG. 4. (A) Silver-stained SDS-PAGE of LPSs from *P. aeruginosa* whole control cells (WCC), whole cells exposed to four times the MIC of gentamicin (WCG), n-MVs, and g-MVs. LPS preparations were made with 40 μg of protein from each of the indicated samples, digested with proteinase K, and resolved in SDS–12% PAGE. (B and C) Comparison of Western immunoblots of this LPS profile reacted with B-band- and A-band specific MAbs, respectively.

Additionally, PLC-H can hydrolyze sphingomyelin but not phosphatidylserine, whereas PLC-N hydrolyzes phosphatidylserine but not sphingomyelin (1, 2, 48). We examined the MVs for hemolytic activity on sheep blood agar plates as well as spectrophotometrically on sheep blood cells and found that both types of MVs were positive. We did not attempt to differentiate between PLC-H and PLC-N in the present study.

Alkaline phosphatase activity was also detected in both MV preparations (Table 1). The total activity present in supernatants was reduced by approximately 50% after removal of MVs from culture supernatants, indicating an association of the enzyme with the vesicles (Table 2). Although most of the PLC and alkaline phosphatase was found within vesicles, both enzyme activities were also detected in cellular extracts after intact cells were treated with toluene, indicating that mature enzymes are accumulated within the cell before their release into the extracellular medium. Cell-associated activity of both enzymes has been reported previously (21, 38, 44, 48).

P. aeruginosa secretes several proteases (18, 29, 30, 54). We examined the secretion of elastase and protease in strain ATCC 19660 since the amounts of both enzymes produced by this strain were found to be larger than those for strain H103. No appreciable amount of proteolytic or elastolytic activity was detected in toluene cellular extracts, indicating the lack of intracellular accumulation of active enzymes (Table 1). This observation was in agreement with earlier work (11, 15, 16, 18, 29, 30). Examination of both types of MVs for protease activity demonstrated the association of active enzyme. On removal of vesicles from cell-free culture supernatants, the total protease activity dropped by 18% in untreated culture supernatants and 25% in gentamicin-treated culture supernatants (Table 2). Since an appreciable amount of activity could also be detected in culture supernatants following the removal of vesicles from cell-free medium, the enzyme is probably released from cells in both soluble and vesicle-associated forms. In contrast, elastolytic activity was detected exclusively in culture supernatants and was not affected by removal of vesicles from cell-free culture supernatants. Previous studies have demonstrated that the enzyme is secreted as a proenzyme that becomes active only as it is released into the supernatant (15, 28). For this

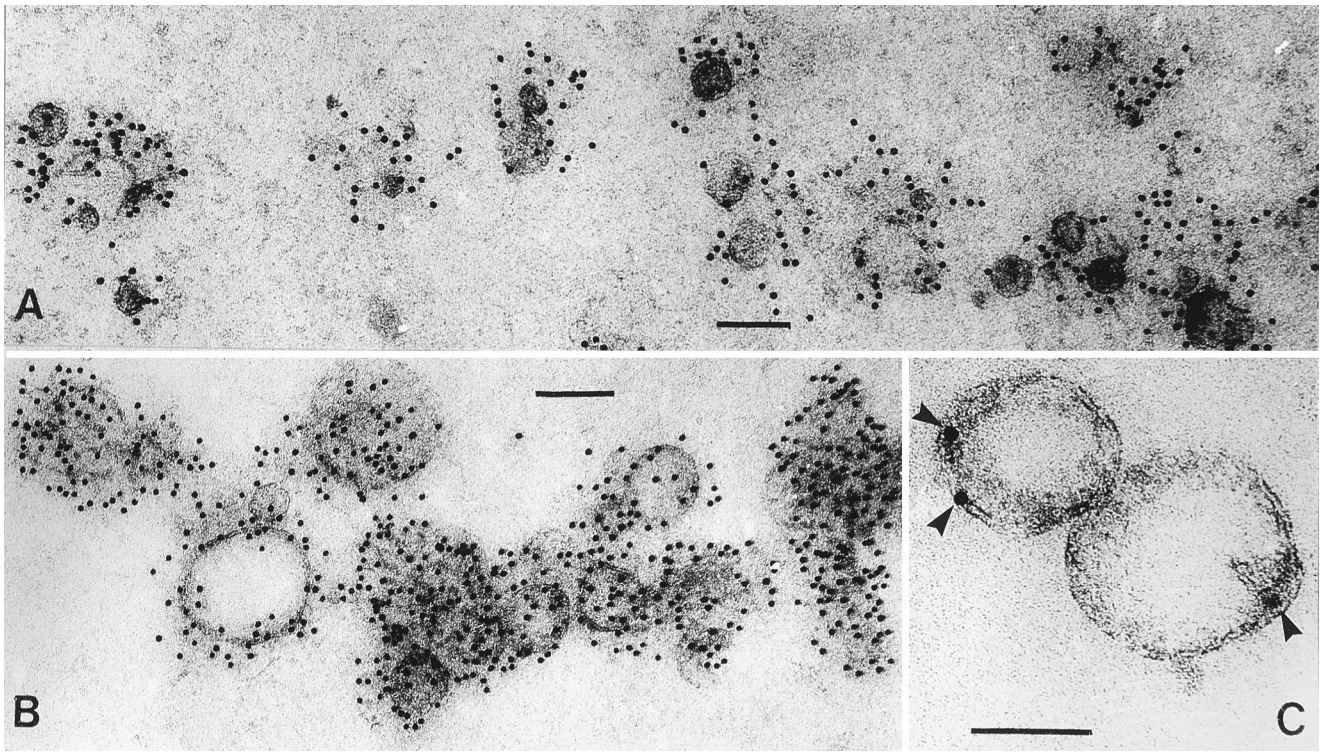

FIG. 5. Immunogold electron microscopic detection of LPS on thin sections of n-MVs (A) and g-MVs (B) with MAb to B-band LPS. Both types of MV appear heavily labelled with antibodies to B-band LPS. However, the degree of labelling was less in n-MVs. Occasionally, g-MVs were labelled with MAb to A-band LPS (C) (arrowheads), but n-MVs were never labelled for this LPS (not shown). The two MAbs labelled whole cells (containing both A- and B-band LPSs) with the same effectiveness. Note that g-MVs are generally larger in diameter than n-MVs. Bars = 100 nm.

TABLE 1. Distribution of extracellular enzymes in MVs and cellular extracts[a]

Sample	Enzyme activity (U/μg of protein)			
	Protease[b]	Elastase[c]	PLC[d]	Alkaline phosphatase[e]
n-MV	1.68 ± 0.26	<0.01	3.45 ± 0.18	1.0 ± 0.02
g-MV	1.69 ± 0.27	<0.01	3.47 ± 0.24	0.85 ± 0.07
WCC[f]	0.07 ± 0.01	<0.01	0.98 ± 0.08	0.71 ± 0.13
WCG[g]	0.08 ± 0.02	<0.01	0.78 ± 0.02	0.47 ± 0.08

[a] All assays were done in triplicate in each of three separate experiments, and the results were averaged. Means ± standard deviations are shown.
[b] Units in Hide powder blue assay. One unit = increase in A_{595} of 0.1/h at 37°C.
[c] Units in elastin Congo red assay. One unit = increase in A_{495} of 0.1/2 h at 37°C.
[d] Units in NPPC assay. One unit = increase in A_{405} of 0.1/h at 37°C.
[e] Units in pNPP assay. One unit = increase in A_{595} of 0.1/h at 37°C.
[f] WCC, control whole-cell extract.
[g] WCG, gentamicin-treated whole-cell extract.

reason, even if the proenzyme is present in MVs, it would not be detectable by its enzyme activity.

Localization of enzymes by immunoelectron microscopy. Immunogold labelling of ultrathin sections with enzyme-specific antibody enabled subcellular visualization of the enzyme in intact cells and MVs. Figure 6A and B show gentamicin-treated cells labelled for PLC. As judged by the location of gold particles on thin sections, a uniform distribution of the enzyme in the cytoplasm is clearly seen. Interestingly, it can also be seen that cytoplasm is streaming into a forming vesicle. Budding and free vesicles were labelled to the same extent with gold particles, demonstrating that PLC is entrapped within both types of MVs. This was in good agreement with our biochemical demonstration of the enzyme activity in vesicle preparations (Tables 1 and 2). Immunogold labelling for the localization of alkaline phosphatase in thin sections of intact cells and MVs demonstrated that the majority of the enzyme was located in the envelope, particularly in the periplasm and outer membrane (Fig. 6C). MVs were labelled on the membrane and on the luminal material attached to the membrane. g-MVs and n-MVs were labelled to approximately the same extent, confirming the result of the enzymatic assay (Table 1).

Little or no antibody was seen in the cytoplasm of thin-sectioned cells labelled for either alkaline protease or elastase (Fig. 6D and E). Gold particles were seen in MVs labelled for protease, but the labelling was not as intense as for the previous enzymes. No MVs were labelled for elastase (Fig. 6D). These results were in good agreement with the bioassay (Table 2). For both elastase and protease, clusters of gold particles were located near the plasma membrane, within the cell envelope, or at the outer membrane surface. These are presumably the sites where the enzyme is being translocated through the envelope for release into the surroundings.

TABLE 2. Enzyme activities in cell-free culture supernatants following the removal of MVs[a]

Enzyme	% Activity remaining in cell-free supernatant after removal of:	
	n-MVs	g-MVs
PLC	32 ± 8	17 ± 5
Alkaline phosphatase	49 ± 7	52 ± 6
Elastase	98 ± 0.8	98 ± 0.9
Protease	82 ± 1.9	75 ± 4

[a] All assays were done in triplicate in each of three separate experiments, and the results were averaged. Means ± standard deviations are shown.

Immunological detection. Western blots of cell extracts, MVs, and culture supernatants before or after treatment with gentamicin were analyzed by SDS-PAGE and probed with either elastase-specific (Fig. 7A) or alkaline protease-specific (Fig. 7B) polyclonal antiserum. Elastase antigen with an apparent molecular mass of 33 kDa, corresponding to the mature elastase from *P. aeruginosa*, was detected in culture supernatants. A faint band corresponding to elastase and two additional bands with molecular masses of ~42 and 45 kDa were seen in both types of MVs (Fig. 7A). Cellular extracts appeared to have two weakly reacting bands, corresponding to ~32 and 45 kDa. These minor bands that were antigenically related to elastase are most likely the larger precursors of active mature elastase. In fact, when we treated these precursors in MVs by the method of Fecycz and Campbell (12) to remove noncovalently bound inhibitor molecules, elastase activity was restored (data not shown).

A major protein band with a molecular mass of 48 kDa was seen in culture supernatants in the Western blot probed with alkaline protease-specific antiserum (Fig. 7B). This is the same apparent molecular mass as that of the protease standard purified from *P. aeruginosa* culture supernatants (30). Although the results from the bioassay with Hide powder blue indicated proteolytic activity associated with MVs (Table 1), Western blot analysis with antibodies specific to alkaline protease did not show an antigenically related band corresponding to the 48-kDa enzyme (Fig. 7B), suggesting that the proteolytic activity seen in the enzyme assay is most probably due to protease(s) other than alkaline protease. The MVs and cell extracts expressed two or three faintly stained bands in the range of 40 to 45 kDa when reacted with alkaline protease-specific antibodies. At present, we do not know whether these antigenically related bands were degradation products or precursors of alkaline protease. However, previous investigators have been unable to detect intracellular proenzyme. Since it has been demonstrated that cell-bound protease degrades rapidly (15), it is possible that these bands represent degradation products which are recognized by the antibodies. Clearly, some labelling is also seen on the thin sections (Fig. 6D). However, the presence of proteinases in MVs was clearly demonstrated on zymogram gels (Fig. 7C). Both MVs are composed of three major proteolytically active polypeptides with different molecular masses (~33, 35, and 135 kDa), suggesting that vesicles indeed possess protease activity, confirming the enzyme assay (Tables 1 and 2).

Alkaline phosphatase occurs in two varieties, high-M_r alkaline phosphatase (H-phosphatase) and low-M_r alkaline phosphatase (L-phosphatase) (44). A single band with an M_r of ~51 was seen in the Western blot when probed with polyclonal antiserum raised against H-phosphatase. No band was seen when the blot was probed with L-phosphatase. The synthesis of H-phosphatase is known to proceed constitutively, whereas L-phosphatase must be induced by phosphatase limitation in the growth medium (44). Since our culture medium used to grow the cells was not phosphate limited, L-phosphatase was not expressed.

Fluorometric quantification of DNA in MVs. To determine whether DNA is packaged within vesicles, MVs from two different strains of *P. aeruginosa* were analyzed for DNA content by using a highly sensitive, DNA-specific assay. As seen in Table 3, DNA was found in both n-MVs and g-MVs from both bacterial strains. More was found in the latter than the former, and strain ATCC 19660 packaged more DNA into MVs than did strain H103. During the assay, DNA must first be liberated

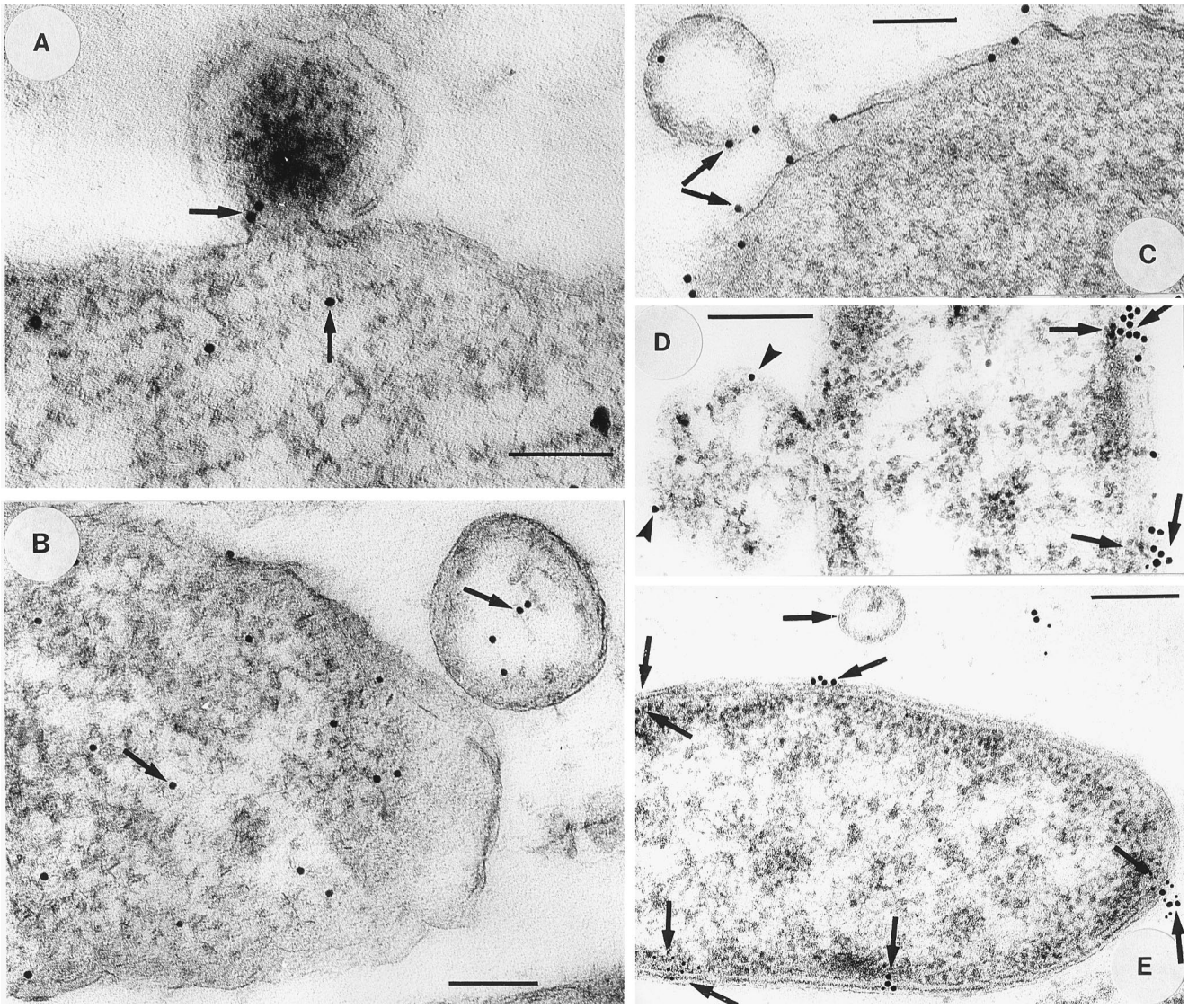

FIG. 6. Immunogold electron microscopic detection of PLC on thin sections of gentamicin-treated whole cells as g-MVs are formed and sloughed off. (A) Formation of a vesicle on the cell surface. The enzyme is labelled in both the cytoplasm and the forming vesicle (arrows). (B) A g-MV has just separated from the cell. The lumen of the MV is labelled, as is the cytoplasm of the cell (arrows). The periplasm was only occasionally labelled, presumably because most PLC was being rapidly packaged into MVs. Soluble extracellular enzyme would not be seen by this technique because of the numerous washing steps involved with fixing and embedding the cells. Similar results were obtained with n-MVs. (C) Immunogold electron microscopic detection of alkaline phosphatase in cells treated with gentamicin. Note that the gold particles are associated with the outer membrane or are in the periplasm of intact cells and on the periphery of the released vesicle (arrows). (D and E) Immunogold electron microscopic detection of alkaline protease and elastase, respectively, in cells treated with gentamicin. Note that a few gold particles are associated with the MV probed for alkaline protease (arrowheads) but not elastase (an MV is indicated with an arrow). A significant subpopulation of gold particles labelling alkaline protease and elastase appears to be located in discrete regions at or near the outer membrane (arrows), indicating the sites where the soluble enzymes are released. Bars = 100 nm.

from vesicles for accurate fluorometric detection. Since some DNA may remain stuck to membrane fragments (as they are being separated from the analysis liquor), ammonium hydroxide–Triton X-100 was used to solubilize MVs for some analyses; the DNA content of all solubilized samples was consistently greater than that found when intact membranes were removed (cf. results for solubilized versus intact MVs in Table 3). This difference could simply be due to the sticky nature of DNA (thereby reflecting indiscriminate membrane binding), or it could reflect a truly membrane-bound fraction. TEM of rotary-shadowed samples showed linear strands resembling DNA in each preparation, but no accurate estimation of membrane association could be made (data not shown). Table 3 also shows that there were clear differences between the amounts of DNA found in the MVs of the two bacterial strains, and it is possible that the degree of DNA entrapment in MVs was strain dependent.

Because the MVs were isolated from early-stationary-phase cultures, it was also possible that some of the DNA was derived from lysed cells (within each culture) which had bound to the outer face of the vesicles. This could especially be true of g-MVs. Control experiments conducted with exhaustive treatment of MVs with pancreatic DNase showed that this was not the case. DNase-treated n-MVs and g-MVs possessed amounts of DNA similar to those in Table 3. These control experiments also confirmed that the MVs were intact, since the DNA of the

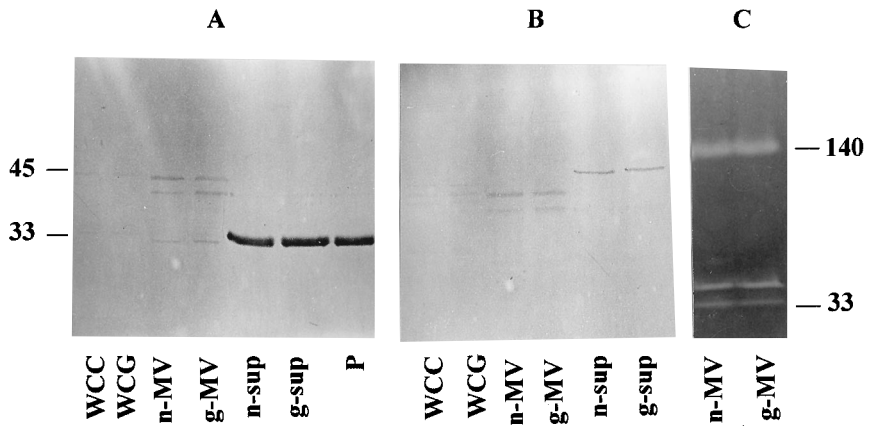

FIG. 7. (A and B) Western immunoblot analyses of samples with antibodies to elastase and alkaline protease, respectively. Whole-cell extracts from untreated control cells (WCC) or gentamicin-treated cells (WCG), n-MVs and g-MVs, and cell-free supernatants after removal of MVs from untreated (n-sup) or gentamicin-treated (g-sup) cultures are shown. Lane P, purified elastase. Each lane contains 25 μg of protein or 10 μl of concentrated n-sup or g-sup. (C) Proteinase present in MVs tested by gelatin zymography. Both n-MVs and g-MVs demonstrated three major bands (~33, 35, and 135 kDa) having proteolytic activity. Molecular masses (in kilodaltons) are indicated on the left and right.

MVs was protected from the external enzyme. When in similar experiments mixtures containing MVs and free DNA were treated with pancreatic DNase and ethidium bromide gel electrophoresis was performed, the external DNA was shown to be digested, whereas the MV DNA remained intact (data not shown).

DISCUSSION

P. aeruginosa releases MVs into the medium during normal growth, and the amount is increased severalfold on exposure of the organism to gentamicin. Naturally evolved vesicles and gentamicin-induced vesicles possessed several important virulence factors. Since outer membrane blebs are commonly excreted from gram-negative cells (23, 33, 35, 51, 52), endotoxins were expected to be present. However, the MVs of *P. aeruginosa* were enriched with a particular variety of LPS from the cell surface, B-band LPS. Furthermore, discrete enzymes such as alkaline phosphatase, PLC, and protease were packaged into both MV types; each of these components is important in the pathogenesis of *Pseudomonas* infections. For example, protease is required for the establishment of corneal infections (19), and PLC acts cooperatively with alkaline phosphatase in liberating P_i from phospholipids (42, 44). PLC from *P. aeruginosa* not only causes paralysis, dermonecrosis, vascular permeability, and death in mice (2), but it also is a potent inflammatory agent (1, 2) and plays an important role in lung infections (2, 14).

TABLE 3. DNA content in MVs derived from two strains of *P. aeruginosa*

Strain	Vesicle type	ng of DNA/mg of protein (mean ± SD)[a]	
		Solubilized[b]	Intact
ATCC 19660	n-MV	18.85 ± 0.63	14.35 ± 0.49
	g-MV	21.44 ± 0.33	16.12 ± 1.02
H103	n-MV	10.56 ± 0.48	7.69 ± 0.32
	g-MV	11.07 ± 0.89	8.87 ± 0.60

[a] $n = 4$ for each estimate.
[b] Solubilized in Triton X-100 and ammonium hydroxide.

Alkaline phosphatase. Almost 20 years ago, Ingram et al. (21) suggested that secreted alkaline phosphatase was associated with LPS. Our results clearly substantiate this by demonstrating the association of the enzyme with MVs which are enriched with LPS. Alkaline phosphatase, although not directly involved in pathogenesis, is a periplasmic marker in many gram-negative bacteria, including *P. aeruginosa* (21, 30, 38, 44), and its release could be controlled by the shedding of MVs. The enzyme is known to be synthesized cooperatively with PLC, scavenging organophosphate (often from membrane phospholipids) as it is released. This entire process is regulated by the concentration of P_i in the medium (30, 42), and choline can be added to support the production of PLC (42). Even higher concentrations of these enzymes would have been expected if the organism had been grown in phosphate-deficient medium (42, 44). We did not conduct experiments under phosphate limitation, since *P. aeruginosa* increases its secretion of LPS under these conditions (21), which would complicate interpretation.

Elastase. Our failure to detect enzymatically active elastase within MVs can be explained by previous work (12, 28). It has been shown that the enzyme is first made as an inactive preproelastase in the cytoplasm and a proelastase in the periplasm (12, 28, 29). This proenzyme becomes modified as it crosses the outer membrane and becomes active. Because MV-entrapped elastase would not have crossed the outer membrane (i.e., it remains periplasmic), the enzyme would remain inactive and would not have been detected by our assay. Furthermore, an inactive elastase with the same molecular weight as the mature elastase has been demonstrated in the periplasmic space of wild-type and mutant strains of *P. aeruginosa* (28, 29). Bever and Iglewski (5) demonstrated that these precursor proteins reacted with elastase-specific antiserum. We also detected higher-M_r elastase-like molecules, using specific antiserum in immunoblots (Fig. 7A), and we restored elastase activity in MVs when inhibitor substituents were removed. Together, these observations indicate that elastase precursors were concentrated within the MVs. Presumably, in order to mature into active elastase, the precursors must remove their inhibitor molecules prior to translocation across the outer membrane of the MVs. In this way, MV elastase must have the same activation requirements as the enzyme which is cell bound within the periplasm.

Two-step secretion and PLC. The involvement of pseudomonal *xcp* gene products in secretion has been demonstrated by the intracellular accumulation of these enzymes in strains that carry mutations in components of the extracellular secretion machinery (13, 30, 54) or by cloning of the genes encoding the enzymes in *E. coli* (5, 30). In *E. coli*, the exoproteins are translocated to the periplasm but are not secreted, suggesting that the specific secretion activities needed to cross the outer membrane are absent in *E. coli*. The *Pseudomonas* mutants may have architectural alterations in their outer membrane which discourage secretion. Indeed, Hamood et al. (16) demonstrated that OMPs of variable size were missing in *P. aeruginosa* mutants. It would be interesting to see if these mutants were defective in MV production. Our preliminary results suggest that *E. coli* K-12 is less efficient than *P. aeruginosa* at releasing MVs.

For elastase, the signal sequence which is important for translocation across the inner membrane is considered to be of a normal length (i.e., 25 amino acids) (5, 30). However, PLC, which is also considered to be secreted via the two-step mechanism, has an unusually long (38-amino-acid) positively charged signal sequence (48). We found the majority of secreted PLC to reside within MVs. Since the second step of this pathway (by which PLC is released from the cell) is not yet fully understood, we believe that a major proportion of PLC is released via MVs.

Protease. Although the secretion of alkaline protease is not affected by the *xcp* gene products, it is, however, affected by the *apr* gene products (13, 54). Translocation across the two membranes is thought to occur via three accessory proteins (AprD, AprE, and AprF) presumably located at adhesion zones. It has been suggested that the protease cleaves holes in the cell envelope to gain direct access to the extracellular medium so that there is no significant intracellular accumulation (13, 30, 40). Because of this self-promoted egress, the enzyme would have only a short, transient time in the cell envelope and would escape detection by current techniques (30), yet the Hide powder blue assay indicated proteolytic activity in both types of vesicles, which was unequivocally confirmed by using zymogram gels. However, no band corresponding to the M_r of alkaline protease was detected in Western blot analysis. This suggests that the activity seen in the enzyme assay was due not to alkaline protease but instead to some other protease. This activity was also not likely due to elastase either, as MVs lacked elastolytic activity until they were chemically activated by cleavage of inhibitor substituents. Other proteases, such as staphylolytic proteases (29) or uncharacterized proteases, may account for the proteolytic activity found in MVs. We are attempting to further characterize this protease activity.

Problems with single- and double-step models for all forms of secretion. None of the current models take into account how these complex exoproteins traverse the peptidoglycan matrix. We have not been able to detect a distinct peptidoglycan layer in MVs (Fig. 1, 2, 5, and 6), but we and others have recently demonstrated that both types of MVs were enriched with peptidoglycan-hydrolyzing enzymes (i.e., autolysins [4]). Certain proteases produced by *P. aeruginosa* are bacteriolytic endopeptidases that could lyse the peptide bridges between peptidoglycan strands (7, 29, 43). Furthermore, we have shown by thin-section electron microscopy the transient disruption of the *P. aeruginosa* peptidoglycan directly beneath outer membrane blebs caused by gentamicin, a gentamicin-bovine serum albumin complex, and amikacin (23, 33, 49). Such strategically placed and limited hydrolytic activities would require the specific activation or deregulation of localized autolysin(s) over short periods. This limited hydrolysis of peptidoglycan would allow the passage of extracellular proteins through the peptidoglycan matrix. Some of the protease activity that was detected in MVs could be due to such autolysins. This is currently under investigation in our laboratory.

For both single- and double-step secretion pathways as currently envisioned, there remain major questions concerning the definition of the steps requiring ATP hydrolysis or membrane potential, the molecular nature of protein recognition and protein transit, and the need for unfolding and refolding during and after translocation (particularly the proteins with carboxy-terminal signals). In fact, some of these exoproteins assume fully folded native conformations, including the formation of interchain disulfide bonds. Translocation of such proteins from the periplasm across the outer membrane would require significant rearrangement or linearization of their structures during passage through a narrow outer membrane channel. Following translocation, the proteins would then have to refold into their native conformation. Alternatively, if native, fully folded polypeptides were released from the periplasm, large pores would be required in the outer membrane. Such pores would have to allow unidirectional transit of a broad class of extracellularly secreted proteins without concomitant release of the general periplasmic contents. Such selective portals have not been demonstrated in gram-negative outer membranes (32).

DNA in MVs. Another interesting finding in this study was the demonstration of DNA within MVs which was protected from hydrolysis by an external DNase. Although the presence of DNA in membrane blebs has been reported for a few other gram-negative bacteria (10, 26), we believe that this is the first report demonstrating the export of DNA material via MVs in *P. aeruginosa*. At present, we are investigating whether these vesicles could serve to transfer genetic material to other strains of *P. aeruginosa* or even other species and genera.

An MV model. On the basis of our present observations, we propose that MVs may also contribute to the export of certain macromolecules, including enzymes and DNA, from *P. aeruginosa* to the external environment (Fig. 8). The export of such molecules involves localized disruption of the cell envelope and would occur naturally during normal growth of the bacterium. The addition of a membrane perturbant, such as gentamicin, would increase the incidence of MVs and possibly affect their overall chemical nature. For example, we believe that gentamicin would increase the cytoplasmic content of some g-MVs, since it could penetrate far enough into the cell envelope to perturb the cytoplasmic membrane. Cytoplasmic membrane enzymatic markers NADH oxidase and succinic dehydrogenase were both found in g-MVs, indicating that this membrane is occasionally breached and cytoplasm is liberated (data not shown).

Figure 8A shows the envelope before membrane blebbing is initiated. Figure 8B shows the simplest type of MV and is the most frequent n-MV. This MV is comparatively small, involves only the exfoliation of the outer membrane, and entraps only periplasm. Figure 8C is an extrapolation of Fig. 8B in that it illustrates the entrapment of DNA that has migrated from the cytoplasm to the periplasm and is another possibility for n-MVs. Although the DNA resembles linear strands, it is possible that both circular and linear complexes could be compartmentalized. Figure 8D shows the production of the most complicated MV, since it contains both inner and outer membranes as well as some cytoplasmic constituents. Autolysins in both n-MVs and g-MVs have been detected (4), and it is presumed that the normal close attachment between the outer membrane and peptidoglycan within the cell envelope requires a concomitant hydrolysis of the peptidoglycan layer immedi-

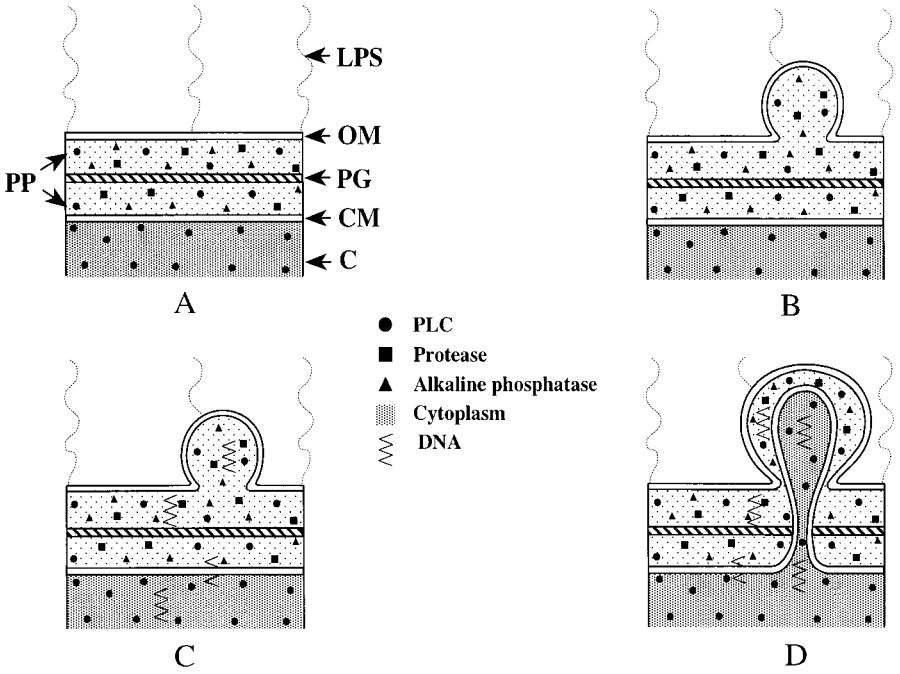

FIG. 8. Model representing events leading to the secretion of certain extracellular enzymes via MVs in *P. aeruginosa*. C, cytoplasm; CM, cytoplasmic membrane or plasma membrane; PG, peptidoglycan layer; OM, outer membrane. n-MVs are developed during normal growth and contain mainly B-band LPS, mature periplasmic extracellular enzymes, and proenzymes. Gentamicin increases the incidence and size of MVs (g-MVs) and frequently penetrates to and perturbs the CM, resulting in vesicles which contain OM, CM, and C. For both n-MVs and g-MVs, the peptidoglycan layer is weakened by autolysins, but since the effect is greater with gentamicin, cell lysis can ensue (17, 23, 25, 33, 49).

ately below the MV during blebbing. This breach in the peptidoglycan during n-MV development would be localized and highly transient, so that cell lysis would not ensue. Because gentamicin increases both the incidence and the size of MVs, autolytic activity must increase and would contribute to cell death (17, 23, 25, 33, 49). As a general membrane perturbant, gentamicin would encourage the formation of the MVs seen in Fig. 8D.

The binding of polycations such as aminoglycosides to *P. aeruginosa* can rearrange the LPS packing order and destabilize the outer membrane (17, 33, 47, 49). Thermodynamically, these regions of the outer membrane would have to reconfigure their constituent macromolecules, resulting in the increased blebbing into the external milieu. Since Mg^{2+} is liberated from the outer membrane during the initial stages of gentamicin treatment (33, 49), a free pool of the cation might become available to activate autolysins, which may account for the increased localized hydrolysis of peptidoglycan directly below a g-MV (23, 33). Alternatively, the actual perturbation of the outer membrane may so rearrange the normal spatial relationship between peptidoglycan and autolysin that the enzymes become activated. Thus, binding of gentamicin to the outer membrane weakens the entire cell envelope, so that both periplasm and cytoplasm can enter the MVs.

We are not denying that other investigators have detected the presence of several extracellular enzymes and LPS of *P. aeruginosa* in culture supernatants (9, 14, 15, 19, 28) and that spontaneously released or antibiotic-induced extracellular products have also been demonstrated in cell-free culture supernatants (9, 20, 37, 39). Yet, unless high-speed centrifugation of the supernatants had cleared them of the MVs seen in our study, substantial so-called extracellular activity would have been retained in the MV particulate fraction and not in a soluble form as many researchers believe. There has been no general appreciation of the particulate nature of these factors' release. Our recognition that a certain percentage of these factors is immobilized in MVs has several ramifications. First, since n-MVs are found in untreated cultures, these vesicles must be a normal consequence of cellular growth and metabolism. It would be unusual if this costly expenditure of cellular substance was simple happenchance; clearly, the vesicles are not recycled, since they are set adrift and do not remain cell associated. For a pathogen such as *P. aeruginosa*, whose virulence and tissue-destructive power reside in its ability to deliver its virulence factors by external means, MVs make sense. They are small and can readily fit into epithelial crevices. They are bilayer and, accordingly, can meld into larger bilayer structures, such as host membranes. Endotoxin is enriched over the MV surface, and certain tissue-destructive enzymes are concentrated within the MVs; MVs should make efficient delivery vehicles for these factors as the pathogen initiates infection. Since more MVs are liberated from the bacterium when it is treated with a surface-active aminoglycoside antibiotic such as gentamicin, it is possible that g-MVs flood the patient's system during antibiotic treatment, causing massive shock to the immune system and targeted localization within certain tissues. This phenomenon could partly contribute to the common toxicity problems associated with these pharmaceutical agents (36, 37).

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