Role of Outer Membrane Vesicles of Bacteria

M V Jagannadham and M K Chattopadhyay

Outer membrane vesicles are nano-sized spheres produced predominantly by gram-negative bacteria. They play a significant role in cell-to-cell communication, virulence, nutrition and protection of the bacterial cells from various stress factors. Recent evidences also underscore their involvement in antibiotic-resistance of bacteria. In this article, we discuss the physiological importance of these vesicles with potential use in the development of vaccines and drug delivery.

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Introduction

Bacteria are unicellular microorganisms but in natural environments, they live as a community with other microorganisms. The formation of consortium calls for a mutualistic relationship to be developed with the other cells. Alternatively, they assume the role of a pathogen inside the body of plants and animals. The secretion of proteins in bacteria plays a significant role in their communication with other microorganisms and also with the host cells. It is also essential for detoxification of chemical stressors (antibiotics, disinfectants), sensing the presence of the nutrients and elimination of competitors. So far, six types of protein secretion systems in gram-negative bacteria have been extensively studied and characterized. The secretion of proteins through outer membrane vesicles (OMVs) is another mechanism, receiving the attention of microbiologists during the past three decades.

OMVs are small spherical bags of 20–300 nm diameters, shed mostly by gram-negative bacteria in the extracellular environment. They are rich in outer membrane proteins and mostly depleted in cytoplasmic contents. They are not products of cell fragmentation or sloughing off the outer membrane but outcome of the normal turnover of the cells. They are not formed by fragmentation of the cell or accidental detachment of some por-

Keywords
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Historical Background

In 1965, D G Bishop and Elizabeth Work at Twyford Laboratories, London observed that a lysine-requiring strain of the colon bacterium, *Escherichia coli*, secreted some extracellular lypoglycopeptide. A modification of the method followed by them in the next study led to the isolation of a soluble lipopolysaccharide complex. Studies involving electron microscopy revealed that the secreted materials were actually outer membranes shed in the form of spheres with an electron-dense centre. The investigators postulated that there was a deficiency of peptidoglycan synthesis in absence of sufficient amount of lysine in the medium and the excess amount of outer membrane synthesized could not remain bound to the cell-wall and shed in the external environment in the form of spherical vesicles.

Subsequently, in the course of their investigations into the mode of the secretion of cholera toxin using electron microscopy, two researchers (S N Chatterjee and J Das) at the Calcutta School of Tropical Medicine observed a bulging of the cell wall and the formation of blebs at places in a *Vibrio cholerae* culture in the logarithmic growth phase. They also found particles resembling the pinched-off membrane sacs in the cell-free culture filtrate. However, the observations reported by them could not be repeated by some other investigators and, therefore, were termed as artefact caused by poor fixation or improper choice of fixative in electron microscopy. The scepticism was bolstered by some other studies. A critical re-examination of the whole process by Chatterjee and his co-workers proved beyond any doubt that bleb formation (protrusion or bulging out of the plasma membrane) indeed took place in the logarithmic phase of growth irrespective of the culture medium – fixative or embedding – used. They also showed that the phenomenon of bleb formation was not associated with cell lysis or abnormal separation between the cell wall and plasma membrane. Bleb formation was subsequently ob-

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served in several other organisms (e.g., *Neisseria meningitidis*) during normal growth conditions. The presence of vesicles was demonstrated in biopsies of tissues infected with various pathogenic bacteria (viz, *Escherichia coli*, *Moraxella catarrhalis*, *Pseudomonas aeruginosa*).

A couple of years back, vesicle formation by some gram-positive bacteria was demonstrated. Vesicle formation by several types of mammalian cells (hematopoietic, epithelial and tumor cells) and archeal cells was also evidenced. Hence, vesicle formation appears to be a phenomenon conserved in various types of living cells.

Biogenesis of OMVs

Though almost 50 years have elapsed since the first report on OMVs was published, the exact nature of the factors that govern the formation of the vesicles remains to be elucidated. A number of ideas on the biogenesis of OMVs are available in the literature with both supportive and contradictory evidences. Vesicles are formed by protrusion of the outer membrane and pinching-off from the membrane. It is a process which requires energy though no ATP or other energy source is available at the site of budding. Bulging of the membrane can occur when the membrane is disrupted. However, studies on *E. coli* revealed that the instability of the membrane is not a prerequisite for vesiculation. It is proposed that the imbalance in the turnover of peptidoglycan leads to accumulation of muramic acid. The hydrostatic pressure generated by osmotic imbalance at places in the periplasm leads to the bulging of the outer membrane. Occurrence of low molecular weight muramic acid in some OMVs corroborates this hypothesis.

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The bulging of the outer membrane also requires delinking of the outer membrane from peptidoglycan. The proteins linking the outer membrane and peptidoglycan may be disrupted or relocated in the process. This idea was supported by the absence of some outer membrane proteins in OMVs produced by *E. coli*. It is

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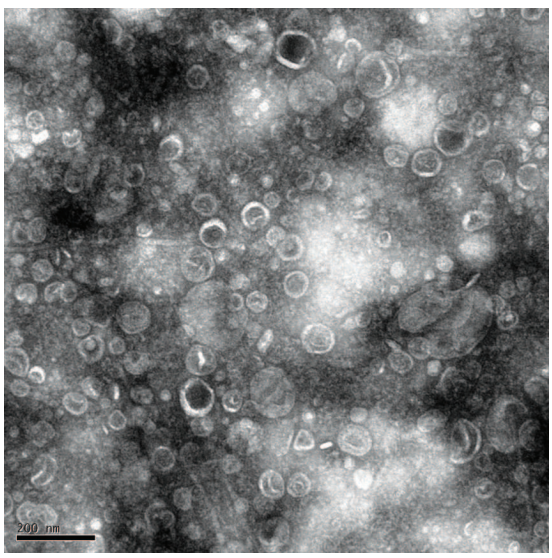
known that OMVs are produced during normal growth. But their formation has been found to be stimulated in the presence of some physical (heat) and chemical (detergents) agents. Although OMVs are produced within the log phase, some OMVs are also formed during the stationary phase, probably due to the fact that some cells in the bacterial population still remain in the log phase. The effect of nutrients on the production of OMVs is also not the same in all bacteria. Vesicle formation was found to be upregulated in low nutrient condition in case of *Lysobacter*, whereas it was enhanced in *Pseudomonas fragi* when nutrients were available.

Studies performed from time to time indicate the role of a number of genes in vesiculation. The Tol-Pal system of *E. coli* consists of a number of envelope proteins. The genes that encode them are organized in two operons. The system is believed to be involved in maintenance of the integrity of the membrane. Mutations in the *Tol-Pal* genes are known to be associated with increased formation of OMVs. Mutation in another gene *nlp I* in *E. coli* was found to be associated with hypervesiculation without having any effect on the integrity of the membrane. Nlp I is a lipoprotein believed to be involved in cell division. But its exact role in vesiculation is not yet known. The evidences obtained so far clearly indicate that the



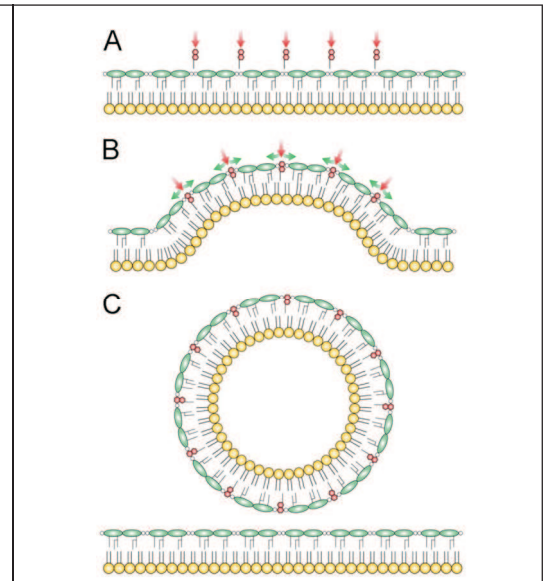


Figure 1 (left). The TEM image of the outermembrane vesicles of *S. typhimurium*.

Figure 2 (right). Bilayer coupled model of vesicle formation in *P. aeruginosa*.

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formation of OMVs is a multifactorial, highly complex phenomenon and extensive studies are required to elucidate its mechanism.

Structural Components

The outer membrane vesicles of bacteria are chiefly composed of proteins and lipids of the outer membrane and periplasm. The number of proteins differs largely in OMVs obtained from various bacterial strains. In general, in *Escherichia coli*, 0.2%–0.5% of the outer membrane and periplasm proteins are packaged into OMVs. Studies performed from time to time have revealed the presence of 6 proteins in OMVs obtained from *P. aeruginosa*, 48 proteins in the OMVs of *N. meningitidis*, 44 proteins in the OMVs produced by *Pseudo altereomonas antarctica* NF3 and 141 proteins in the OMVs of *E. coli*. The porins[1] or tarnsmembrane channel proteins are one of the components of OMVs. Among the other proteins, the virulence factors constitute a major fraction. The different types of virulence factors detected in OMVs produced by various pathogenic bacteria include enzymes (protease, acid phosphatase, β-glucuronidase, lipase, urease, cellulase, chitinase), molecular chaperones (Hsp 60), toxins (shiga toxin, heat labile enterotoxin of enterotoxic *E. coli*, cholera toxin), fimbriae and many other type of proteins.

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[1] Porins are water-filled channels or pores responsible for the transport of small metabolites (e.g sugars, amino acids, ions) through the membrane. They are also found to occur in the OMVs.

The composition of the OMVs produced by the same organism also varies with presence and absence of stress. Bacteria under different stress conditions were found to partition more proteins into the vesicles. Growing evidences suggest that cytoplasmic proteins and genetic material like DNA and RNA are also packaged into the vesicles.

Among the lipids, phospholipids and lipopolysaccharides are the major components that are found to occur in OMVs. Occurrence of lipoproteins is also observed in OMVs in some cases. The lipid composition of the OMVs from *Pseudomonas syringae* Pv tomato T, studied at our laboratory, revealed the presence of phosphatidylethanol amine and phosphatydylglycerol with varying lengths of fatty acyl chains.

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Functional Importance

Role in Secretion

OMVs are formed through an energy-requiring process. Hence, their formation and secretion must have some biological significance. It has not been possible to isolate any gram-negative bacterium or its mutant which does not produce OMVs. So vesicle formation might have an indispensible role in survival of the organisms. It appears that bacteria use OMVs as a sort of special secretory device.

There are several features which make them distinct from the other secretory systems. Both soluble and insoluble proteins can be transported to their targets, located far away from the cell, through OMVs. Packaging of proteins into OMVs offers a number of advantages. They remain protected from proteases that might occur in the extracellular environment. Moreover, multiple proteins can be delivered at the target site at a time in required concentration, without being diluted in the extracellular fluid.

A number of models have been proposed so far to explain how OMVs deliver their content into the different types of target cells. It is postulated that OMVs lyse spontaneously to release their content in the vicinity of the target cells. The OMVs are known to be stable and the spontaneous lysis of OMVs is a rare phenomenon. However, the OMVs released by some gram-negative bacteria were found to lyse various gram-positive and gram-negative bacteria by a number of investigators. The vesicles are believed to attach themselves to the cell surface of the gram-positive bacteria and break open to release the enzyme peptidoglycan hydrolase, which disrupts the underlying cell-wall. In case of gram-negative bacteria, the vesicles are believed to fuse with the outer membrane of the target cells, release their luminal content in the periplasm, where the enzyme peptidoglycan hydrolase can diffuse around and attack the peptidoglycan in a number of sites.

While interacting with eukaryotic cells (e.g., during pathogen-

esis), bacterial vesicles are believed to attach themselves to the host cells through some adhesive molecules on their surface and subsequently enter the host cells through endocytosis. For example, in the case of enterotoxigenic *Escherichia coli* (ETEC) infection, a heat labile enterotoxin (LT), bound to the lipopolysaccharide of the vesicles, gets attached to the GM1 receptor of the host cells. Binding to this receptor is associated with internalization. This is how the vesicles produced by the ETEC appear to enter the intestinal cells. Similarly, a vacuolating toxin produced by *Helicobacter pylori* is associated with the surface of the OMVs produced by the pathogen. Binding of the vesicles to the host cells and subsequently endocytosis of the vesicles are believed to be mediated through the toxin. However, not all the surface-associated bacterial toxins mediate binding and internalization of the vesicles.

The OMVs are important for pathogenicity and virulence of bacteria.

Role in Pathogenesis

OMVs are important for pathogenicity and virulence of bacteria. Studies involving various pathogenic bacteria clearly reveal that they produce OMVs within the infected host tissues. Body fluids (blood, urine) collected from the infected patients in the clinical setup and also from the infected animals in the laboratory are found to contain vesicles, thus indicating migration of OMVs from the site of infection.

Vesicle production in pathogenic strains was found to be several times greater compared to their non-pathogenic counterparts in a number of investigations. Because of their small size, vesicles produced by a pathogenic organism can reach and interact with the host tissues, which are otherwise inaccessible to the bacterium. Some time back, it was demonstrated that the gram-positive bacterium *Bacillus anthracis* formed OMVs containing toxins and anthrolysin, which were delivered to the host cell. Multivalent adhesins, required for colonization of host tissues by gram-negative bacteria, can be efficiently delivered at the target through OMVs. Both pathogenic and non-pathogenic bacteria produce vesicles for survival inside the host. In non-pathogenic bacteria,

Abundance of the vesicles is observed in biofilms formed *in vivo* or *in vitro*, thus indicating possible role of OMVs in biofilm formation.

A quorum sensing molecule produced by *Pseudomonas aeruginosa* (Pseudomonas Quinolone Signal, PQS) is hydrophobic and therefore requires the help of OMVs to be transported from cell-to-cell. Formation of OMVs in this organism on the other hand, is dependent on the synthesis of PQS.

they may work by sequestering the attacking bacteriophages.

Role in Biofilm Formation

Biofilms are aggregates of bacteria embedded in a polysaccharide matrix produced by the bacteria. Naturally occurring biofilms observed in various places (e.g., within water pipes or on masts of the ships) harbour multispecies populations. Biofilms formed within the hosts (on urinary catheters, cardiac pacemakers, heart valve replacement, artificial joints and other surgical implants) provide a haven for the pathogenic bacteria; they remain resistant to the antibiotics compared to their free-living counterparts. An abundance of the vesicles is observed in biofilms formed *in vivo* or *in vitro*, thus indicating a possible role of OMVs in biofilm formation. They are believed to mediate co-aggregation of bacteria. HmuY, a unique protein produced by *Porphyromonas gingivalis*, the causative agent of chronic periodontitis, helps the organism to acquire the heme protein and in the formation of the biofilm. This protein was found to be associated with OMVs. The DNA molecules present on the surface of some OMVs are also believed to serve as a bridging component in biofilms. OMVs can also nucleate the process of biofilm formation.

Role in Cell-to-Cell Communication

Bacteria communicate among themselves by releasing some soluble chemicals (autoinducers), which are detected by other members of the community, leading to the realization of coordinated behaviour. The amount of autoinducers produced is proportionate to the population size. Thus, autoinducers indicate that the population size has reached a certain threshold (quorum). The phenomenon called quorum sensing is crucially important in biofilm formation, production of virulence factors and antibiotics and also in other physiological processes. While some of the autoinducers are water soluble, a quorum sensing molecule (2-heptyl-3-hydroxy-4-quinolone) produced by *Pseudomonas aeruginosa* called Pseudomonas Quinolone Signal (PQS) is hydrophobic and, therefore, requires the help of OMVs to be

transported from cell-to-cell. Formation of OMVs in this organism on the other hand, is dependent on the synthesis of PQS. Addition of exogenous PQS to a PQS-deficient culture of *P. aeruginosa* and also to other gram-negative bacteria, leads to the formation of OMVs. Thus it is ensured that the PQS, notwithstanding its hydrophobic nature, reaches its target after formation.

Role in Self Defence

Antibiotics are chemical substances of microbial or synthetic origin. They suppress the growth of or kill microorganisms other than the producer. Hence, antibiotics pose a threat to bacteria. However, bacteria are known to evolve with different types of strategies that make them immune to the growth-inhibitory or killer effects of antibiotics. Recent evidences indicate that the membrane vesicles protect bacteria against some antibiotics. In a study involving a hyper-vesiculating mutant of the wild-type *E. coli*, two antibiotics acting on the outer membrane (polymixin B and colistin), could not prevent the growth of the mutant. Formation of the vesicles was found to be significantly induced in the presence of the antimicrobial peptides. Growth-inhibitory effect of two membrane-active antibiotics on an Antarctic bacterium was found to be reversed at our laboratory upon addition of OMVs isolated from the same organism, to the culture medium. Uptake of the antibiotics by the vesicles was also demonstrated by an assay involving fluorescent-labelled compounds. Thus, the vesicles appeared to protect the bacterial cells by removing the antibiotics from the medium and not allowing them to reach their target.

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On the other hand, presence of ciprofloxacin in the vesicles produced a ciprofloxacin-resistant mutant of the mycoplasma *Acholeplasma laidlawii* reported some time back, indicates that the vesicles might protect the organism by transporting the antibiotic from inside to the outside of the cells, thus not allowing it to accumulate in sufficient concentration required for its activity. In some cases the OMVs are found to carry the enzyme

β-lactamase, which converts β-lactam antibiotics (penicillins and cephalosporins) into therapeutically inactive compounds. The vesicle-transported enzyme is observed to protect not only the producer organism but also some other bacteria known to co-occur with the OMV-producer in the human respiratory tract. The ability of the vesicles to shield the enzyme β-lactamase from inactivation by anti-β-lactamase antibody has been demonstrated in an *in vitro* study.

In another investigation, two clinical isolates of the bacterium *Acinetobacter baumannii*, a major cause of healthcare-associated infections, were found to produce OMVs containing the plasmid-borne $bla_{OXA\text{-}24}$ gene, which encodes a β-lactamase responsible for carbapenem-resistance. A carbapenem-sensitive strain of the organism was found to turn resistant following incubation with these vesicles. Subsequently, it started releasing OMVs containing the resistance-conferring gene. So in this case, OMVs played the role of a vector for horizontal transfer of the resistance-conferring gene. Thus, OMVs appear to work through multifaceted mechanisms for mediation of antibiotic-resistance in bacteria.

Protective action of OMVs was also demonstrated against the T4 bacteriophage by some other investigators. Besides helping bacteria in containing the challenge posed by the antibiotics and bacteriophages, OMVs also appear to play a role in competition with other bacteria in the extracellular milieu for food. In a nutrient-deficient environment, OMVs produced by *P. aeruginosa* were found to carry the cell wall degrading enzyme called peptidoglycan hydrolase, which could lyse other bacteria occurring nearby.

Role in Stress Tolerance

OMVs also relieve bacterial cells of the stress, caused by the accumulation of misfolded proteins.

Stress is a relative term. Any condition an organism is not normally habituated to endure is a stress for it. Thus, even an aerobic environment is stressful to an obligate anaerobe. However, in a general sense, stress implies extremities of temperature,

pressure, pH, salinity, desiccation and radiation. Presence of high concentration of oxidative substances and toxic chemicals, paucity of essential nutrients in the environment and presence of misfolded protein within the cell also exert stress on bacteria.

Proteins, which guide the enzyme RNA polymerase to transcribe the right gene when required, are called sigma factors. It is well known that in response to various types of stress conditions, synthesis of different sigma factors is induced in bacteria. Genes transcribed by them encode proteins, which work in various ways ultimately to relieve the cells of stress. The Sigma E protein is involved in the management of extracytoplasmic stress. It is activated by another protein DegS. In a study conducted at the Duke University Medical Center (Durham, USA) involving the bacterial strain *Escherichia coli* DH5α, the rate of vesicle formation was found to be enhanced when the temperature was increased. A very high level of vesicle production was observed in a mutant having its *degS* gene inactivated by transposon mutagenesis[2]. In absence of the DegS protein in this mutant, the stress-relieving effect of the Sigma E was also absent and improper folding of proteins caused by increase in temperature led to cellular stress. Thus, it was evidenced that OMVs also relieve bacterial cells of the stress, caused by the accumulation of misfolded proteins. The investigators also got evidence of a specific packaging mechanism that enabled the misfolded proteins to be incorporated into the OMVs and transported out of the cell. This feature (removal of the undesirable materials from the cell) makes the vesicles analogous to the membrane-bound microparticles of the eukaryotic cells, which serve the same purpose. Overexpression of periplasmic protein also is known to stimulate vesicle production. Increase in vesiculation at high temperature might also be an indirect effect of high temperature, which increases the growth rate leading to enhanced production of OMVs.

[2] A technique that uses a traceable mobile genetic element to randomly disrupt genes in the chromosome.

Increase in vesiculation is also observed, when bacteria are exposed to various chemical stressors. In a recent investigation,

OMVs need not necessarily contain DNA inside the lumen and internalize DNA fragments into the target cells. The same purpose might be achieved if DNA is carried on the surface of OMVs.

production of OMVs was found to be enhanced in *Pseudomonas aeruginosa* following treatment with D-cycloserine (a known inhibitor of peptidoglycan synthesis), polymixin B (an antibiotic which acts on the outer membrane of bacteria) and hydrogen peroxide (which imparts oxidative stress inside the bacterial cell). Unlike what was found earlier in *E. coli*, vesicle formation was not found to increase in *P. aeruginosa* with increase in temperature. Thus, it is evident that mechanism used for counteracting the same stressor, varies in different gram-negative organisms.

Role in Gene Transfer

OMVs are known to facilitate horizontal gene transfer (HGT, movement of genetic material between different species) by carrying DNA fragments. An example of the ability of the vesicles to facilitate HGT leading to the dissemination of antibiotic resistance in bacteria is highlighted in the relevant portion of this article. However, OMVs need not necessarily contain DNA inside the lumen and internalize DNA fragments into the target cells. The same purpose might be achieved if DNA is carried on the surface of OMVs.

Role in Procurement of Nutrients

Some enzymes (e.g., aminopeptidase) transported by the OMVs help the producer organism in acquiring nutrients from the surrounding. OMVs also carry receptors that acquire nutrients. For example, the pseudomonas quinolone signal (PQS) may act as a siderophore to fetch iron from the surrounding. The PQS-Fe-OMVs complex delivers iron by being absorbed into the outer membrane by fusion. The complex may also dissociate to release the PQS-bound iron near the cell. Thus, OMVs promote survival of the producer cell in iron-limiting environments, which is most often encountered by pathogenic bacteria inside the host. In an investigation performed a couple of years back, indication was obtained of possible involvement of OMVs in enzymatic reduction and transformation of heavy metals and radionuclides. It was

OMVs also carry receptors that acquire nutrients.

observed that OMVs produced by the metal-reducing bacterium *Shewanella* contained proteins and cytochromes that are essential for coupling the oxidation of hydrogen to the reduction of multivalent metals; an indication that the OMVs carried some terminal reductase was also obtained.

The ability of OMVs to deliver bacterial toxin in a concentrated form to the host cell makes them a potential candidate for the preparation of vaccines.

Biotechnological Potential of OMVs

The ability of OMVs to deliver bacterial toxin in a concentrated form to the host cells makes them a potential candidate for the preparation of vaccines. They are immunogenic, self-adjuvant and easily taken up by the mammalian cells. Hence, several attempts have been made to prepare vaccines against various diseases using OMVs. Cerebrospinal meningitis (most often referred to as meningococcal disease) is a recurrent problem in the developing countries. OMV-vaccines were found to be protective against serogroup B meningococcal outbreak. Subcutaneous injection of OMVs obtained from *Burkholderia pseudomallei* (the causative agent of melioidosis, a disease responsible for significant morbidity and mortality in Southeast Asia and Northern Australia) was found to be protective against the lethal challenge by *B. pseudomallei* in aerosol in BALB/c mice.

Pasteurella multocida along with *Mannheimia haemolytica* is a major cause of bovine respiratory disease. Following intranasal immunization of BALB/c mice using OMVs obtained from these two organisms, humoral and mucosal immune response were evidenced by enzyme-linked immunosorbent assays (ELISA). OMV-immunization was also found to confer immunity against *Vibrio cholerae* in a rabbit model. These and many other reports in the literature underscore the immense potential of OMVs as a component of vaccines. The vesicle-based vaccines promise safety as they do not contain live bacteria.

Keeping in mind the involvement of OMVs in pathogenesis and antibiotic-resistance of bacteria, it appears worthwhile to look into the therapeutic potential of the inhibitors of vesicle formation to control bacterial infections.

In a study reported a couple of years back, production of OMVs was found to be enhanced in *Shigella flexneri* in presence of gentamicin. The antibiotic-induced vesicles were observed to package the antibiotic, penetrate through *S. flexneri*-infected

It appears feasible to reconstitute the vesicles with biologically important proteins and to improve their ability to transport the proteins to the host cells.

cultured human intestinal epithelial cells and kill the intracellular bacteria following delivery of the antibiotic. Gentamicin and other aminoglycoside antibiotics cannot penetrate through mammalian cell membrane and therefore cannot be used to treat infections caused by intracellular pathogens (e.g., *Shigella* spp, *Salmonell*a spp). This study provides a clue to overcome the problem by using OMVs as a vehicle for drug delivery. In some cases, it has also been possible to transform bacterial cells with DNA packaged into OMVs. So there is multifaceted scope of using OMVs as biotechnological tool. Keeping in mind the involvement of OMVs in pathogenesis and antibiotic-resistance of bacteria, it appears worthwhile to look into the therapeutic potential of the inhibitors of vesicle formation to control bacterial infections.

Conclusion

The various roles of OMVs in bacterial physiology offer an interesting field for investigation. Recent reports on the involvement of OMVs in protection against stress add a new dimension to the present state of knowledge on stress adaptation of bacteria. It has been already mentioned that vesicles are produced also by the archeal and eukaryotic cells. Hence, studies on OMVs are likely to provide insight into one of the basic biological processes that occur in all forms of life. The OMVs are known to interact with both eukaryotic and prokaryotic cells and deliver the contents into the host cells. Taking clues form the identification of different components present in the OMVs, it appears feasible to reconstitute the vesicles with biologically important proteins and to improve their ability to transport the proteins to the host cells.

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