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EXPERIMENTAL ARTICLES

Outer Membrane Nanovesicles of Gram-Negative Bacteria *Aeromonas hydrophila* and *Aeromonas salmonicida*

K. A. Lusta[1] and Yu. E. Kozlovskii

Scientific Research Institute of Human Morphology, Russian Academy of Medical Sciences, ul. Tsuryupy 3, Moscow, 117418 Russia

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Abstract—*Aeromonas hydrophila* and *A. salmonicida* grown in pure cultures were found to secrete extracellular membrane nanovesicles into the environment. Outer membrane nanovesicle preparations were isolated by differential centrifugation and ultrafiltration and visualized by transmission electron microscopy applying the negative staining technique. Membrane nanovesicle size (10–300 nm) and ultrastructure were determined. The vesicles were especially numerous around bacteria at the edge of small colonies. The process of the vesicle budding off the bacterial cell was observed. On ultrathin sections of rat intestine, outer membrane nanovesicles were revealed among bacterial aggregates of various species of parietal microorganisms. Short chains of such vesicles were also detected inside the glycocalyx between the microvilli of the apical surface of the intestine epitheliocytes. On the basis of the results, together with the literature data, the secretion of the outer membrane nanovesicles by pathogenic gram-negative bacteria, such as *A. hydrophila* and *A. salmonicida*, is proposed as a possible mechanism of pathogenesis leading to the host disease, as well as a means for cellular interactions both within the prokaryote population and between the bacteria and the host organism.

Keywords: outer membrane nanovesicles, gram-negative bacteria, *Aeromonas hydrophila, Aeromonas salmonicida*, transmission electron microscopy, intercellular interactions.

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Recent works have shown that a number of gram-negative bacteria secrete into the environment the outer membrane nanovesicles (OMNVs), formed by the bacterial outer membrane and containing components of the periplasm [1]. OMNVs secreted by pathogenic bacteria were established to contain virulence factors and may deliver them to the host organism (see table).

All gram-negative bacteria that have been studied to date produce the OM nanovesicles naturally [22]. Using the radioactively labeled proteins and toxins, native OMNVs produced by bacteria were demonstrated to contain as much as 12% of the total cellular material [23]. Generally, OM nanovesicles reflect the composition of the outer cell membrane of gram-negative bacteria and contain lipopolysaccharides, glycerophospholipids, and proteins of the outer membrane, as well as the components of the periplasm and cytoplasm [1, 8]. In addition, fragments of plasmid and chromosome DNA, as well as bacteriophage DNA, may be packed in the OMNVs [24].

Detailed proteome analysis of the OMNVs from various bacteria species carried out by two-dimensional electrophoresis and mass spectrometry revealed over 100 proteins in the isolated vesicles [12, 21], including proteins of the bacterial outer membrane (AcfD lipoprotein precursor, outer membrane receptor protein TolC, ferrichrome protein precursor, outer membrane antigen, phospholipase A1 precursor, Tsx channel-forming protein, LC outer membrane porin precursor, colicin I receptor, vitamin B_{12} receptor precursor, cytolytic toxins, etc.), periplasmic proteins (Protease DegQ precursor, the periplasmic glutamine-binding protein, β-glucosidase, a lipoprotein carrier protein, sulfatase precursor (YdeN), a cytotoxic metalloprotease, etc.), and cytoplasmic proteins (chaperone protein HtpG, alkyl hydroperoxide reductase C22, elongation factor Tu, succinate dehydrogenase flavoprotein subunit, dihydrolipoamide dehydrogenase, dihydrolipoamide succinyl transferase component, etc.).

However, it is not known at present whether such vesicles are produced by gram-negative bacteria of the genus *Aeromonas*. *Aeromonas hydrophila* and *A. salmonicida* are rather common in the environment and may cause diseases in fish, amphibians, mollusks, and human beings [25]. For example, *A. salmonicida* causes furunculosis and hemorrhagic bacteremia in many fish species, leading to losses in fish population in freshwater basins and fish farms [26]. In human beings, infection by *Aeromonas*, especially *A. hydrophila*, leads to the development of such inflammatory diseases as sepsis, meningitis, pneumonia, peritonitis, conjunctivitis, corneal ulcer, osteomyelitis, arthroem-

[1] Corresponding author; e-mail: k_lusta@rambler.ru.

Virulence factors revealed in the outer membrane nanovesicles of various bacteria

Bacterial species	Virulence factor	Reference
Actinobacillus pleuropneumoniae	Proteases, ApxI	[2]
Actinobacillus actinomycetemcomitans	Leukotoxin	[3]
Bacteroides fragilis	Hemagglutinin, hydrolytic enzymes	[4]
Bordetella pertussis	AC-Hly toxin, hemaglutinin (FHA), pertussis toxin (Ptx)	[5]
Borrelia burgdorferi	OspA, OspB, OspD toxins	[6]
Burkholderia cepacia	PLC-N, lipase, PSCP, a 40-kDa protease	[7]
E. coli (ETEC)	LT toxin	[8]
E. coli (STEC)	Shiga toxin	[9]
E. coli (EHEC)	ClyA cytotoxin	[10]
Helicobacter pylori	VacA toxin	[11]
Legionella pneumophila	Mip (Ipg0791), IcmK/IcmX, LaiE/LaiF, hydrolytic enzymes	[12]
Moraxella catarrhalis	UspA1/UspA2	[13]
Neisseria meningitidis	PorA, NlpB, NarE	[14]
Pseudomonas aeruginosa	Hemolysin, hydrolytic enzymes, Cif and PQS toxins	[15]
Salmonella typhi	ClyA cytotoxin	[10]
Shigella flexneri	IpaB, IpaC, IpaD toxins	[16]
Shigella dysenteriae	Shiga toxin	[17]
Treponema denticola	Proteases, dentilysin	[18]
Vibrio anguillarum	Metalloproteinase, hemolysin, and phospholipase	[19]
Vibrio cholerae	RTX toxin	[20]
Xanthomonas campestris	Cellulase, glucosydase, xylosidase, and nonvirulent proteins	[21]

pyesis, myositis, liver abscess, cholecystitis, biliary tract inflammation, urinary infections, endocarditis, ear, nose and throat infections, etc. [27].

Pathogenic properties of these bacteria are determined by the production of a number of virulence factors, including enzymes like lipase, gelatinase, and hemolysins, as well as cytotoxins and enterotoxins (the membrane-damaging α- and β-hemolysins, cytolytic enterotoxin, a pore-forming toxin aerolysin, thermostable and thermolabile enterotoxins, and thermostable and thermolabile metalloproteinases) [28]. The course of the infectious processes caused by *Aeromonas* bacteria is poorly studied, and the mechanisms of toxin secretion in the form of OM nanovesicles and their further interaction with host cells are unknown.

Electron microscopic study of nonpathogenic and pathogenic strains of aeromonads may provide valuable data for better understanding of the mechanisms of intercellular interactions and pathogenesis upon the bacterial infection. The goal of the present work was, therefore, to study the ultrastructural organization of cells and outer membrane nanovesicles of *A. hydrophila* and *A. salmonicida* in pure cultures, as well as in rat parietal intestine microflora cells.

MATERIALS AND METHODS

Microorganisms. The nonpathogenic strain *Aeromonas hydrophila* 342-1 obtained in the Laboratory of Molecular Microecology, Scientific Research Institute of Human Morphology, Russian Academy of Medical Sciences, together with three pathogenic strains—E 8-8, H 336, and H 1-6-05—from the collection of the Laboratory of Genetically Engineered Preparations (Scientific Research Institute of Fur Animals and Rabbit Breeding, Russia), and an *A. salmonicida* A-450 strain from the collection of the University of Guelph, Canada, were used in the work.

Cultivation. All *A. hyfrophila* strains were grown on LB agar plates. For electron microscopy, they were cultured at 37°C for 20 h. *A. salmonicida* A-450 was grown on Trypticase soy agar plates at 22°C until small colonies were formed. In order to isolate the outer membrane vesicles formed by these bacteria, the cultures were grown on trypticase soy broth (TSB) (Difco Laboratories, Detroit, United States) in Erlenmeyer flasks at 22°C with continuous agitation on a rotary shaker at 125 rpm to the onset of the stationary growth phase.

Transmission electron microscopy. Small individual bacterial colonies (~0.5 mm in diameter) grown on agarized media were enrobed in melted 2% (wt/vol) agar. Upon agar solidification, whole colonies were

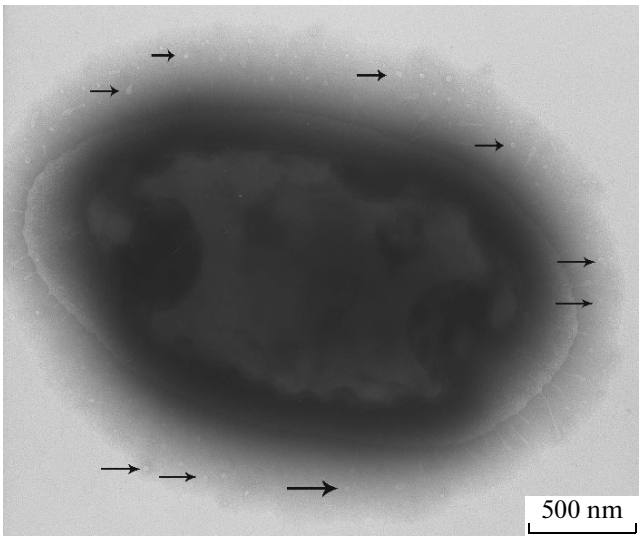

Fig. 1. Transmission electron microscopy of *A. salmonicida* A-450 cells. Negative staining with ammonium molybdate. Agglomerates of numerous bubbles 10–50 nm in diameter are revealed on the bacterial cell surface.

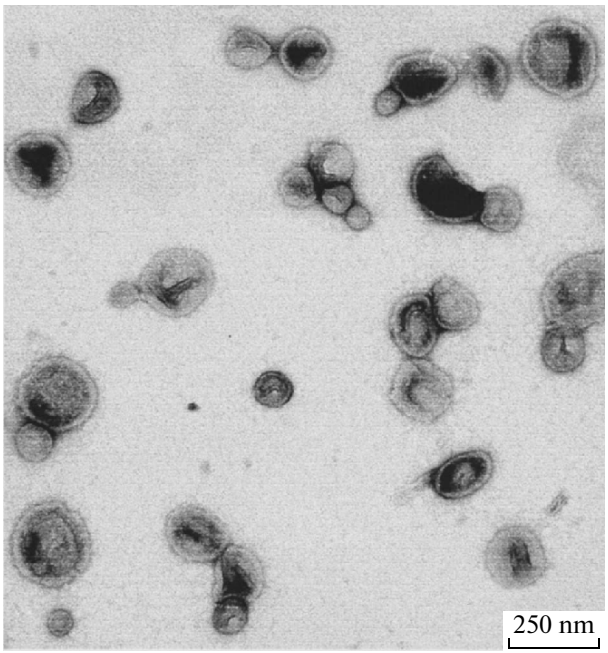

Fig. 2. Membrane nanovesicles isolated by differential centrifugation and ultrafiltration from *A. salmonicida* A-450. Negative staining with uranyl acetate.

cut out by a lancet. These preparations were fixed for 1.5 h at 4°C with a solution containing 0.5% (vol/vol) glutaraldehyde and 2.5% (vol/vol) formaldehyde in 100 mM sodium phosphate-buffered saline (PBS), pH 7.2, washed with the buffer three times for 5 min, and then fixed in 1% OsO_4 solution in the same buffer for 1.5 h (4°C). After fixation, the material was dehydrated in alcohols of increasing concentration, namely, 50%, for 15 min; 70%, two times for 30 min; and 96%, for 15 min. The samples were then infiltrated in the LR White resin (Fluka) according to the protocol 96% ethanol–resin, 1 : 1, 1 h under shaking; 100% resin overnight under shaking; and another portion of resin for 1 h under shaking. The infiltrated material was embedded in capsules of LR White resin containing the catalyst and polymerized under UV irradiation at 4°C for 1 day. Ultrathin sections were obtained using an LKB ultramicrotome and stained with uranyl acetate and lead citrate according to Reynolds.

For negative staining, 20 μl of the bacterial cell suspension or isolated OM nanovesicles preparations were placed on nickel grids covered with a carbon-coated formvar film and stained with 2% uranyl acetate and ammonium molybdate.

The sample grids were examined with a Zeiss Libra 200 transmission electron microscope.

OMNV isolation. Culture of *A. salmonicida* A-450 was grown on trypticase soy broth (Difco Laboratories) at 22°C with continuous agitation on a rotary shaker at 125 rpm to the early stationary phase. Whole bacterial cells were removed by centrifugation at 6000 *g* for 30 min. The supernatant was filtered once through 0.45- and 0.22-μm cellulose acetate membranes, then through two more 0.22-μm membranes to remove any remaining cells. The filtrate was then ultracentrifuged (150000 *g*, 5°C, 1.5 h). OMNV pellets were washed and resuspended in 50 mM HEPES (pH 6.8) and then stored at –20°C.

RESULTS AND DISCUSSION

The population of *A. salmonicida* A-450 grown in liquid medium was studied by transmission electron microscopy with ammonium molybdate negative staining. Numerous vesicles from 10 to 50 nm in diameter were detected on the surface of the cells (Fig. 1). Suspension cultures of the bacteria were used to obtain preparations of the membrane nanovesicles by differential centrifugation and ultrafiltration. The preparations were visualized by negative staining and transmission electron microscopy. The OMNV fraction was found to be heterogeneous with vesicle size varying from 20 to 300 nm (Fig. 2).

Populations of the *A. hydrophila* and *A. salmonicida* strains grown on LB agar or Trypticase soy agar plates, respectively, were also studied by electron microscopy. Visualization of the ultrathin sections of the cells located close to the surface of small colonies of both pathogenic and nonpathogenic strains revealed membrane vesicles surrounded by a bilayer membrane (Figs. 3 and 4). The most numerous vesicles were observed close to bacteria at the edge of the small colonies. Some of the images depict the process of the vesicle budding off the bacterial cell (Fig. 5).

Thus, gram-negative bacteria *A. hydrophila* and *A. salmonicida* were shown to produce membrane

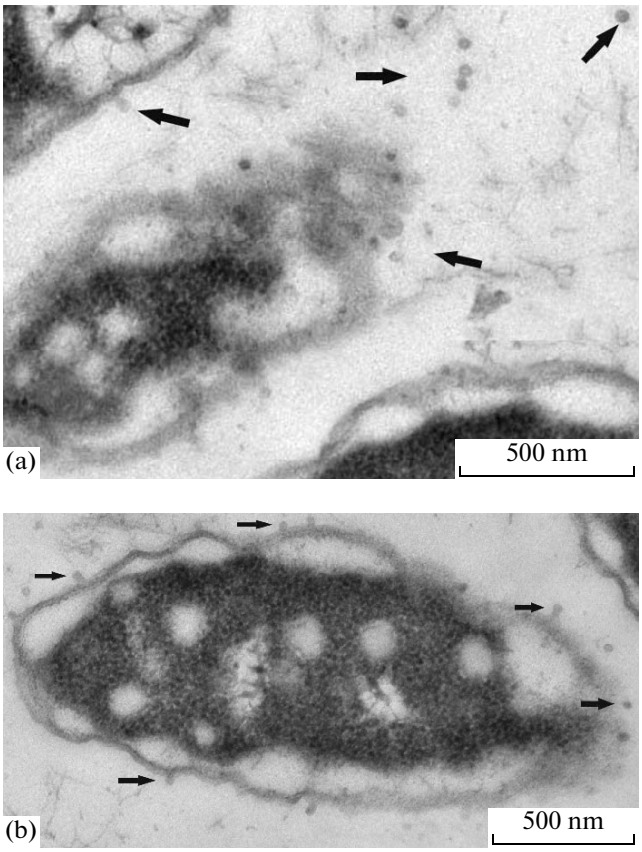

Fig. 3. Ultrathin sections of the cells of the pathogenic *A. hydrophila* strains H 1-6-05 (a) and E-8-8 (b) close to the colony surface. The process of secretion of the membrane vesicles (20–40 nm in diameter) into the medium is demonstrated.

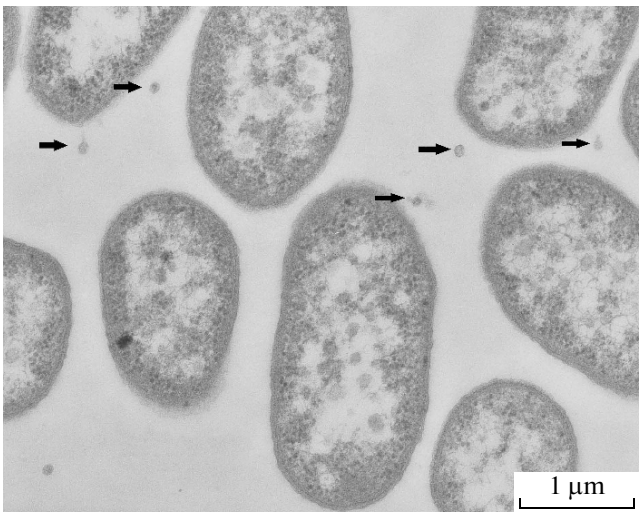

Fig. 4. Ultrathin section of *A. salmonicida* A-450 cells disposed close to the colony surface.

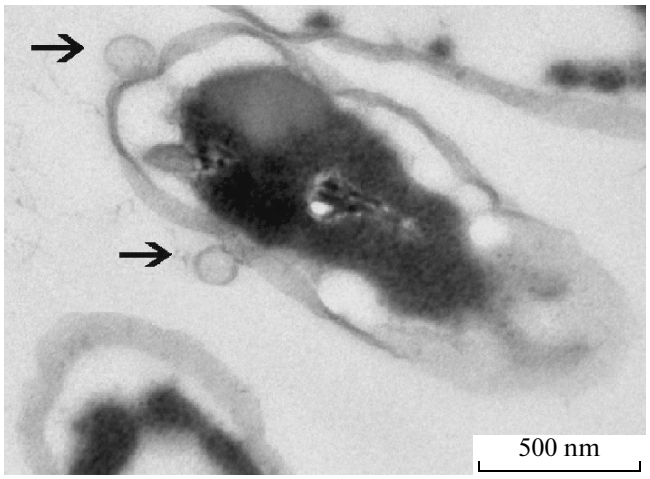

Fig. 5. Ultrathin section of a cell of the nonpathogenic strain *A. hydrophila* 342-1 demonstrating the process of budding of the membrane vesicles (150 nm in diameter) off the cell wall.

nanovesicles different in size and similar to those revealed previously for many other species of gram-negative bacteria (table).

The rest of the work was devoted to the microscopic studies of intestinal microflora. Various bacteria of unidentified species were clearly seen in ultrathin sections of different parts of rat intestine. The most numerous microbial populations were detected on the surface of the intestine walls at the microinvagination sites. Agglomerations of membrane nanovesicles heterogeneous in size were revealed in close proximity to bacterial cells (Fig. 6).

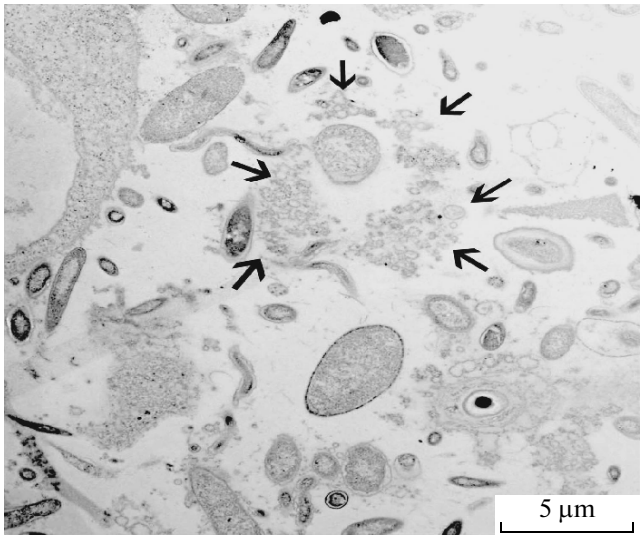

Fig. 6. Ultrathin section of the rat small intestine. Various types and forms of the parietal microbial flora can be seen. Arrows indicate outer membrane nanovesicles.

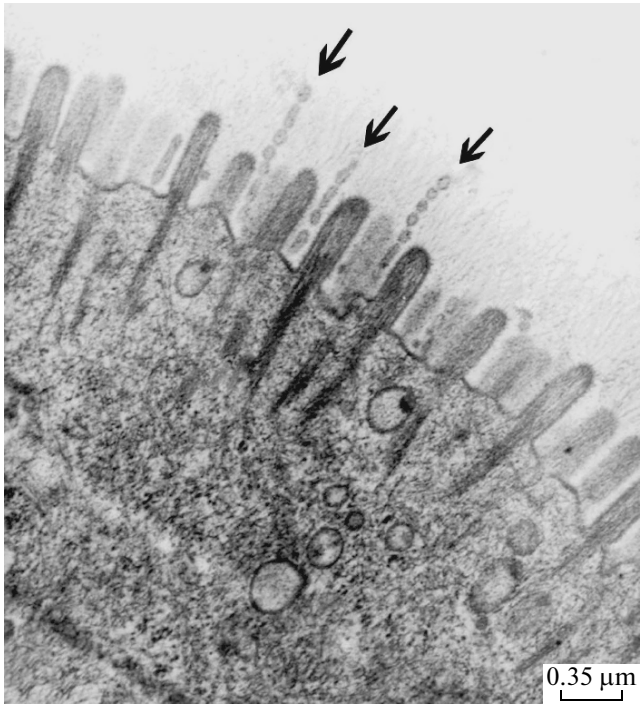

Fig. 7. Ultrathin section of the rat small intestine. Arrows indicate outer membrane nanovesicles.

Free nanovesicles residing independently of cells were detected in ultrathin sections of the rat intestines as chains of several bubbles reaching through the glycocalyx and microvilli of the apical surface of epitheliocytes (Fig. 7). The nanovesicles were not attributed to any bacterial species. Additional experiments are required to identify the OMNVs observed in the preparations by electron microscopy and to elucidate the virulence factors contained therein.

Thus, electron microscopic studies demonstrated that *A. hydrophila* and *A. salmonicida* grown in pure cultures on agar plates and liquid media secrete numerous membrane nanovesicles into the environment. The OMNV size varies between 10 and 300 nm in diameter. Isolated OMNV preparations were obtained and characterized by electron microscopy. It is important to note that similar vesicles were also detected in rat intestines among the agglomerates of the parietal microorganisms of various species and the process of the vesicle penetration through the glycocalyx between the microvilli of the apical surface of epitheliocytes was observed.

Considering the data reported in recent publications on the content of virulence factors, as well as enzymes and DNA fragments in the OMNVs of gram-negative bacteria, membrane nanovesicles produced by the pathogenic bacteria *A. hydrophila* and *A. salmonicida* may be proposed as a mechanism of pathogenesis upon infection by these bacteria. It is of great significance that OMNV outflow by bacteria is another secretion type in addition to the common six types of multiprotein complex secretion by bacteria [29]. The fact of OM nanovesicles' production by both pathogenic and nonpathogenic bacterial strains may evidence that the vesicle functions are not limited to the transport of virulence factors. It is probable that OMNVs are also a tool for communication of gram-negative bacteria with other cells of prokaryotes and eukaryotes.

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