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Molecular characterization of outer membrane vesicles released from *Acinetobacter radioresistens* and their potential roles in pathogenesis

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ABSTRACT

Acinetobacter radioresistens is an important member of genus *Acinetobacter* from a clinical point of view. In the present study, we report that a clinical isolate of *A. radioresistens* releases outer membrane vesicles (OMVs) under *in vitro* growth conditions. OMVs were released in distinctive size ranges with diameters from 10 to 150 nm as measured by the dynamic light scattering (DLS) technique. Additionally, proteins associated with or present into OMVs were identified using LC-ESI-MS/MS. A total of 71 proteins derived from cytosolic, cell membrane, periplasmic space, outer membrane (OM), extracellular and undetermined locations were found in OMVs. The initial characterization of the OMV proteome revealed a correlation of some proteins to biofilm, quorum sensing, oxidative stress tolerance, and cytotoxicity functions. Thus, the OMVs of *A. radioresistens* are suggested to play a role in biofilm augmentation and virulence possibly by inducing apoptosis.

1. Introduction

Outer membrane vesicles (OMVs) are formed by bulging of the outer membrane (OM). This phenomenon makes the neck of OM so constricted that a membrane sac is formed followed by pinching off from cell surface [1]. The process of releasing OMVs is referred to as vesiculation and is not due to the bacterial cell lysis [2,3]. It occurs in both growth and stationary phase [3]. Stress can induce vesiculation as the level of protein accumulation in the periplasm gives rise to a periplasmic pressure [4] followed by stress in cell envelope [5] that subsequently increases vesicle production and also affect the dimensions of the released OMVs [6]. Naturally produced vesicles ranges in size from 50 to 250 nm [5], and often strain dependent [7,8]. OMVs are bilayered [9], the OM of bacterial cell becomes OM of vesicles and periplasm as lumen. Vesicles contain OMPs, periplasmic proteins, phospholipids, LPS, enzymes, genetic material, as well as toxins and other virulence associated factors [8].

OMVs when released in planktonic cells, perform various functions such as horizontal transfer of nucleic acid which is an effective way for bacterial cells to acquire resistance genes against antibiotics [6], defence by delivery of toxins, transfer of nutrients [9], as well as adsorption of antibiotic peptides [10,11] for the survival of bacterial population [11]. They have been detected in the matrix of *Helicobacter pylori* and found enhancing biofilm [12]. OMVs that are released from gram negative bacteria, possess cytotoxic properties also when present *in vivo*, hence contributing to pathogenesis [13]. As OMVs play multifaceted roles, they have been studied for their protein content in many bacterial spp. [14], for instance *Pseudomonas syringae* [15], *Pseudomonas aeruginosa* [16], *Escherichia coli* [17], *Brucella melitensis* [18], *Staphylococcus aureus* [19], *Mannheimia haemolytica* [20], *Xanthomonas campestris pv. campestris* [21], as well as *Acinetobacter baumannii* [22–24].

Acinetobacter is commonly found in the environment and hospitals [25,26]. It is one of the Gram negative bacteria found also on healthy human skin microbiome [27–30] as well as upper

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respiratory tract of humans [31]. It is important to note that antibiotic and metal resistant members of this genus are known for their robust biofilm forming capacity [32,33] and contribute to the pathogenicity [34]. Additionally, members of *Acinetobacter* posses the capacity of rapidly acquiring drug resistance [35–37]. Recent studies have revealed their potential of releasing OMVs in extracellular milieu that has helped *A. baumannii* and *baylyi* in attaining resistance against carbapenem and β-lactamase antibiotics respectively [38,6]. *A. baumannii* has also been reported to be associated with pathogenesis by releasing vesicles containing virulence factors and delivering it to the cytoplasm of host cells [23]. *Acinetobacter radioresistens* is an important member of *Acinetobacter* as it is a silent source of carbapenem resistance [39]. According to recent study, *A. radioresistens* acquires carbapenem resistance due to IS-mediated over expression of intrinsic OXA-23 because of which it is likely that the resistance might spread in the future [40]. It has also been reported to be associated with community acquired bacteremia in HIV positive patient [41]. Moreover, it is a common member of *Acinetobacter* spp. in hospital environmental samples [42], skin of healthy individuals [43], as well as in meat [44]. Despite the fact, *A. radioresistens* has been less explored with respect to the production of OMVs.

Here, we report that *A. radioresistens* releases OMVs under *in vitro* growth conditions. OMVs were further characterized by their hydrodynamic diameter, membrane composition and protein content. Proteome identification and functional analysis of OMVs influenced us to investigate further their possible biological roles in biofilm formation and virulence.

2. Material and methods

2.1. Bacterial strain and growth conditions

A. radioresistens (MMC5 strain) was isolated from a wound infection. The species level identification was done by chromosomal DNA transformation assay, API 32 GN System [29,45] and confirmed by *16SrRNA* gene sequencing (GenBank accession: KC107828). The bacterium was grown and maintained on cysteine-lactose electrolyte deficient (C.L.E.D.) agar and in Luria Bertani broth (LB) (HiMedia, India) at 37 °C with shaking.

2.2. Isolation and purification of OMVs

OMVs were isolated from liquid cultures of MMC5 as previously described [46,23] with some modifications. Briefly, 10 ml of an overnight culture was inoculated into 1 L of LB broth (pH 7.5) and incubated for 15 h at 37 °C with shaking at 150 rpm. Bacterial cells were removed by centrifugation at early stationary phase at 9000 rpm, 4 °C for 30 min (Sorvall centrifuge, rotor SS-34). Supernatant was re-centrifuged at 15,000× *g* for 20 min at 4 °C followed by vacuum filtration using Fast PES bottle top filter (0.2 μm size, Thermo Scientific). The filtrate was concentrated using 10000 MWCO (10 kDa) Vivaspin centrifugal concentrators (Vivascience, Hannover, Germany). OMVs were recovered from the concentrates by ultracentrifugation using Optima™ MAX-XP table top ultracentrifuge (Beckman Coulter, USA) with the rotor TL-100.3 at 30,000 rpm for 90 min at 4 °C. The pellet was washed with HEPES buffer (50 mM, pH 6.8), resuspended in same buffer and filtered through 0.22 μm pore-size syringe filters (Pall Corporation)followed by protein quantification [38] by Bradford protein estimation kit (Bio Rad, USA) according to the manufacturer's manual. OMVs isolated and purified by this method were further used for protein precipitation, separation and identification [47], whereas, other batches of OMVs were treated with Proteinase K (Sigma, Germany) (final concentration 0.1 μg/ml) to digest proteins from the bacterial origin and any phage coats, if present, along with DNase (Bangalore genei, India) (final concentration 10 μg/ml) to digest bacterial DNA. The suspensions were incubated at 37 °C for 10 min followed by deactivating of Proteinase K and DNase at 65 °C for 5 min. OMVs purified by these treatments were used for checking cytotoxicity and biofilm augmentation. One hundred microliters of the vesicle suspension was plated out on LB agar, as well as inoculated into fresh LB followed by incubation for 24 h at 37 °C to confirm the absence of viable cells. In addition, the bacterial cultures were also checked for the presence of bacteriophages according to previously described methods before isolation of OMVs [48].

2.3. Transmission electron microscopy (TEM)

Ten microliters (0.06 μg/ml) of purified OMVs were negatively stained with freshly prepared 3% uranyl acetate for 1 min on 300 mesh-size formvar carbon coated copper grids (Electron Microscopy Sciences, USA). Excess of stain was blotted and the grid was washed once with distilled water and dried. Micrographs were obtained by screening around 30 fields of each grid (triplicates) from three independent batches of OMVs with a high-resolution transmission electron microscope (HRTEM, JEOL-JEM-2100, Peabody, MA) at 100 kV.

2.4. Particle size characterization

Purified vesicles were diluted with HEPES buffer (50 mM, pH 6.8) to 0.06 μg/ml and further filtered through 0.22 μm filters in a particle free environment. The size distribution analysis was performed and recorded by Photocor instrument (College Park, MD, USA) at 90° angle with a laser of wavelength 632 nm. The data was analyzed by Dynals software to obtain the average hydrodynamic radius of given particle. The measurements were conducted at room temperature (37 °C) with 40–50 runs for total 30 min each for every sample and the average intensity weighted diameter was calculated. The average diameter was obtained for OMVs isolated from three independent batches.

2.5. Fatty acid analysis (FAME)

To characterize OMVs, FAME analysis was performed. Fatty acids from three independent batches of overnight grown stationary bacterial cell cultures and purified OMVs of proteins concentrations 220 μg/ml were extracted and transformed into fatty acid methyl esters (FAMEs) by Sherlock MIS protocol [49]. The FAME's were further identified by Gas chromatography (GC) (Agilent 7890A) with an autosampler, an Agilent 7683B injector and Flame Ionization Detector (FID). Helium was used as the carrier gas with the column (Varian Wcot Fused Silica, 50 mx 0, 25 mm (ID). Temperature program used in the column oven was as follows: Starting point: 80 °C for 10 min followed by 3 °C/min to 160 °C for 10 min, 3 °C/min to 200 °C for 15 min and finally 2 °C/min to 250 °C for 15 min. The injection volume was 5 μl and the inlet and the detector were held at a temperature of 260 °C. The column flow was kept 1 ml/min and samples were run on split mode with the ratio 5:1. The FAMEs were identified and qualified by the EZ Chrom Elite software using Supelco 37 component FAME mix as fatty acid standards.

2.6. SDS-PAGE and in-gel digestion

The proteins from OMVs of *A. radioresistens* (MMC5) were obtained from TCA-acetone precipitation method and separated on a 12% SDS-PAGE gel according to the Laemmli's protocol [50]. The protein samples (60 μg) were reduced, alkylated, dissolved in