Microbiological Research 181 (2015) 1–7

Contents lists available at ScienceDirect

Microbiological Research

journal homepage: www.elsevier.com/locate/micres

Protective role of *E. coli* outer membrane vesicles against antibiotics

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ARTICLE INFO

Article history:
Received 24 March 2015
Received in revised form 17 July 2015
Accepted 24 July 2015
Available online 1 August 2015

Keywords:
E. coli
Outer membrane vesicles
Proteomics
MALDI TOF/TOF
Antibiotic resistance
Common defense

ABSTRACT

The outer membrane vesicles (OMVs) from bacteria are known to posses both defensive and protective functions and thus participate in community related functions. In the present study, outer membrane vesicles have been shown to protect the producer bacterium and two other bacterial species from the growth inhibitory effects of some antibiotics. The OMVs isolated from *E. coli* MG1655 protected the bacteria against membrane-active antibiotics colistin, melittin. The OMVs of *E. coli* MG1655 could also protect *P. aeruginosa* NCTC6751 and *A. radiodioresistens* MMC5 against these membrane-active antibiotics. However, OMVs could not protect any of these bacteria against the other antibiotics ciprofloxacin, streptomycin and trimethoprim. Hence, OMVs appears to protect the bacterial community against membrane-active antibiotics and not other antibiotics, which have different mechanism of actions. The OMVs of *E. coli* MG1655 sequester the antibiotic colistin, whereas their protein components degrade the antimicrobial peptide melittin. Proteomic analysis of OMVs revealed the presence of proteases and peptidases which appear to be involved in this process. Thus, the protection of bacteria by OMVs against antibiotics is situation dependent and the mechanism differs for different situations. These studies suggest that OMVs of bacteria form a common defense for the bacterial community against specific antibiotics.

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Introduction

Gram-negative bacteria secrete membrane vesicles of size ranging from 20 to 250 nm (Kulp and Kuehn 2010). Initially, they were found to originate from the outer membrane and were subsequently called as Outer Membrane Vesicles (OMVs) (Bishop and Work, 1965; Work et al. 1966; Chatterjee and Das 1967). Studies on the structural components of these vesicles revealed presence of phospholipids, lipopolysaccharides(LPS), proteins and in some cases nucleic acids (Beveridge 1999; Beveridge and Kadurugamuwa 1996; Sahu et al. 2012; Tashiro et al. 2011; Renelli et al. 2004). The comparison between the structural components of OMVs and their parent bacterial cells hinted towards a selective sorting of these biomolecules. The selective sorting of cytosolic, inner, outer membrane and periplasmic proteins has been observed in the case of proteins (Lee et al. 2008) while in case of LPS too, selective enrichment has been noted (Chowdhury and Jagannadham 2013; Li et al. 1996).

The roles of OMVs as assistants in biofilm formation (Schooling and Beveridge 2006), carriers of virulence and signaling factors (Mashburn and Whiteley 2005), killers of competing microbes (Li et al. 1998; MacDonald and Kuehn 2012), and warheads of an infection process (Schertzer and Whiteley, 2013) have been established. OMVs have been shown to co-ordinate group activities and behavior in bacterial populations (Mashburn and Whiteley 2005). The presence of antibiotics in the medium and stress caused by host environment resulted in increased vesiculation in some bacterial strains (Dutta et al. 2004; Irazoqui et al. 2010). The OMVs were also found to protect the bacterial cells from antibiotic stress when challenged with lethal concentrations of membrane perturbing peptides (Manning and Kuehn 2011; Kulkarni et al. 2014). Multiple functions of OMVs such as toxin delivery to host, lyses of other bacteria, interspecie's communication from a *Pseudomonas* sp. have been reported (Tashiro et al. 2012). Thus, OMVs possess both offensive and defensive functions (MacDonald and Kuehn 2012). Even though some earlier studies revealed that OMVs protect the producer bacterium from antibiotics, the extent and mode of protection is seldom studied in detail.

Among mixed populations, which bacteria are protected against antibiotics, and which are not protected is ambiguous. Hence, it is of interest to investigate whether OMVs have any specificity or bias to protect a particular bacterium against specific type of antibacterial molecules.

In the present study, OMVs from *E. coli* MG1655 were shown to protect the producer bacterium and two other bacteria

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http://dx.doi.org/10.1016/j.micres.2015.07.008
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Pseudomonas aeruginosa and *Acinetobacter radioresistens* from the growth inhibitory effects of membrane-active antibiotics. However, the OMVs of *E. coli* could not protect any of these bacteria from other antibiotics ciprofloxacin, streptomycin and trimethoprim. The OMVs of *E. coli* were found to protect the bacterium either by sequestering the membrane-active antibacterial molecules, or by degrading them.

Materials and methods

The antimicrobials colistin (mixture of polymyxin E1 and E2), melittin, ciprofloxacin, streptomycin, trimethoprim, monosodium phosphate, disodium phosphate, MALDI matrix α-cyano 4-hydroxycinnamic acid (CHCA) were purchased from Sigma. The culture media components yeast extract and peptone used were purchased from Himedia (Mumbai, India). The organic solvents were of UV spectroscopy grade and were obtained from Spectrochem (Mumbai, India).

Bacterial Strains and growth conditions

Three Gram-negative bacterial strains *Escherichia coli* MG1655 (human gut microflora), *P. aeruginosa* NCTC 6751 (Human pulmonary and skin microflora) and an environmental bacterium *Acinetobacter radioresistens* MMC5 were used in this study. All these strains were maintained and grown on LB agar or LB broth at 37 °C with aeration. The strain *A. radioresistense* MMC5 was a generous gift from Dr. B.A. Chopade from University of Pune, India.

Determining minimum inhibitory concentrations

The antimicrobials colistin, melittin, ciprofloxacin, streptomycin and trimethoprim stocks were prepared as described earlier (Andrews 2001). Antibacterial molecules with different mode of action such as those that act on membranes, and others that are either inhibitors of protein synthesis and inhibitors of DNA replication were chosen to study the role of OMVs in preventing their activity. The cationic peptide melittin is is a potent membrane-active antimicrobial agent and has been studied extensively (Asthana et al. 2004). The minimum inhibiting concentrations of the selected antibiotics were determined by broth dilution method modified for 96 well plates (Andrews 2001). LB medium inoculated with the bacterial strains was distributed in different wells. Different concentrations of antibacterial molecules were added to the bacterial cultures. The minimum concentration of the antibiotic molecule where the bacteria fails to grow was estimated as MIC. The growth inhibiting concentrations (above MIC) for colistin, melittin, ciprofloxacin, streptomycin and trimethoprim were used to study the effect of OMVs from *E. coli*. The OMVs of *E. coli* were also used to study their effect of different antibacterial molecules on *P. aeruginosa* and *A. radioresistens*.

Preparation of OMVs from *E. coli* MG1655

The OMVs were prepared from *E. coli* MG1655 by using the method described in the literature (Lee et al. 2007) with minor modifications. In brief, the *E. coli* culture was grown up to stationary phase (up to 18 h of incubation) in LB broth incubated at 37 °C with constant shaking (180 rpm). The cells were separated from the culture by centrifugation at 10,000 × *g* for 10 min at 4 °C. The supernatant was filtered by using 0.45 micron membrane (Millipore). The OMVs in the filtrate were pelleted down by ultracentrifugation at approximately 150,000 × *g* (35,000 rpm for 'type Ti 45' rotor, Beckman) for three hrs at 4 °C in Beckman Ultracentrifuge, in polycarbonate tubes provided by Beckman. The pellet of OMVs was obtained which was subsequently re-suspended in 10 mM phosphate buffer (pH 7.4). This preparation of OMVs was further purified by sucrose density gradient centrifugation. In polyallomer tubes, equal layers of sucrose solutions (prepared in 10 mM phosphate buffer pH 7.4), 70%, 60%, and 20% were added from the bottom to top. The suspension of OMVs was layered on the top of it. The tubes were ultracentrifuged at 35,000 rpm (160,000 × *g* for 'SW 60 Ti' rotor, Beckman) at 4 °C for 4 h. All the fractions were collected from the gradient. Aliquot from each of them was diluted in 10 mM phosphate buffer (pH 7.4) 25 times. They all were tested for the presence of OMVs by using dynamic light scattering (DLS). The fractions that were detected to contain OMVs were pooled together, diluted to 4 mL and subjected to ultracentrifuge at 50,000 rpm (160,000 × *g* for 'TLA-100.3' rotor, Beckman) at 4 °C for 2 h. The pellet of purified OMVs was resuspended in 10 mM phosphate buffer (pH 7.4) and stored at −20 °C until used for experiments.

Quantification of OMVs

The OMVs were quantified in terms of their protein and lipid content per CFUs (colony forming units). The protein content was determined by using Bio-rad Bradford proteins estimation kit and lipid content was measured by using FM4-64 fluorescence (Kulkarni et al. 2014). The number of CFUs in the culture being harvested was determined by dilution plating and colony counting.

Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS) studies

OMVs were prepared from *E. coli* MG1665. DLS was used to estimate the size distribution of these OMVs. The OMVs were diluted to 0. 04 μg/mL in 10 mM phosphate buffer pH 7.4. The scatter was recorded using a Horiba SZ-100 particle size analyzer. TEM was carried out for the OMVs using a Jeol transmission electron microscope (JEM 2100, Tokyo, Japan) at 200 kV. The sample was loaded onto a carbon coated copper grid, negatively stained with 0.2% uranyl acetate.

Growth curve experiments

The change in the growth patterns of all three bacterial strains caused by the addition of OMVs of *E. coli* MG1655, in the presence of growth inhibiting concentrations of antibiotics was monitored in this experiment. All three bacterial cultures were prepared in different wells of 96-well plate as described in the qualitative plate assays ($n = 3$). The growth curve for each of the cultures was recorded by using Spectramax 190 plate reader from Molecular Devices (California, US). The plates were incubated at 37 °C with constant shaking and the OD was recorded at 600 nm at 20 min time intervals. The experiment was carried out in triplicate and a representative growth profile was plotted as time vs. OD at 600 nm The growth of each bacterial strain was studied in the presence of antibiotic with increasing concentration of OMVs.

Quantitative plate assay was performed as described earlier by incubating the bacteria at 37 °C (Kulkarni et al. 2014). This assay was carried out on LB agar plates to study the effect of OMVs of *E. coli* on the growth of *E. coli*, *P. aeruginosa* and *A. radioresistens* in the presence of different antibiotics. In a 96-well plate, the culture medium, inoculums, antibiotics and OMVs were added as described above in the growth curve experiments. In brief, the positive control contained only inoculated medium (10^4 CFU/mL) with growth inhibiting concentration of the respective antibiotic. Increasing concentrations of *E .coli* OMVs were added to the wells. For qualitative studies, the constituents of the wells were streaked on 80 mm LB agar plate segmented in to eight sections. The plates were

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