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Killing Effect of Membrane Vesicles Produced by Gram-negative Bacteria on Other Bacteria

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Abstract: The gram-negative bacteria *Citrobacter freundii*, *Enterobacter cloacae* NCTC10005, *Erwinia carotovora* NCPPB312, *Klebsiella pneumoniae*, *Proteus vulgaris* 1753 and *Serratia marcescens* HIM 307-2 produced natural outer membrane vesicles under normal growth conditions. The membrane vesicles showed bacteriolytic activities against different gram-positive and gram-negative host bacteria. Different killing potencies were obtained by membrane vesicles of different producing organisms against different recipient host strains. In most of membrane-vesicle-producing strains, the exposure to the β-lactam antibiotic cefotaxime and the aminoglycoside antibiotic gentamicin induced the formation of cefotaxime membrane vesicles and gentamicin membrane vesicles, respectively, larger in size and with higher lytic activities against the susceptible host bacteria compared to those produced under normal growth conditions. But the transmission electron microscopy and the plate assay showed that cefotaxime inhibited the formation of membrane vesicles by *E. cloacae* NCTC10005. Natural membrane vesicles produced by *Serratia marcescens* HIM 307-2 and *P. vulgaris* 1753 recorded the widest killing spectrum compared to other natural membrane vesicles. Cefotaxime membrane vesicles of *K. pneumoniae* showed the highest lytic potency, while *C. freundii* membrane vesicles exhibited the least lytic spectrum. The cefotaxime membrane vesicles produced by *K. pneumoniae* had the largest size of 200 nm followed by natural membrane vesicles of *P. vulgaris* 1753 which had a size of 125 nm, while the smallest membrane vesicles were formed by *Serratia marcescens* HIM 307-2 grown under normal conditions. The membrane vesicles produced by different gram-negative bacteria used in this study had spherical shapes and sizes ranged from 30 to 200 nm.

Key words: Gram-negative bacteria, outer membrane vesicles, bacteriolysis, cefotaxime, gentamicin

INTRODUCTION

Gram-negative cell walls have a dynamic feature that is not seen in their gram-positive counterparts, outer Membrane Vesicles (MVs) are constantly being discharged from the cell surface during bacterial growth. MVs are probably the result of the budding (extrusion) of the outer cell membrane of gram-negative bacteria. These structures, ranging in size from 20 to 500 nm, can either be attached to or released from the bacterial cell surface. They have been alternatively called vesicles, microvesicles, blebs, outer membrane fragments, membrane vesicles or extracellular vesicles[1]. The phenomenon of vesicle formation may also result from the fact that the outer membrane grows more rapidly than the underlying peptidoglycan layer[2]. Hayashi *et al.*[3] constructed an autolysin mutant of *Porphyromonas gingivalis* and the mutant produced elevated levels of MVs compared to parental strain. They suggested that vesicle formation of this organism might be regulated by cell wall turnover.

Gram-negative bacterial periplasm normally contains autolysins which along with penicillin-binding proteins are used to help fabricate the peptidoglycan layer as a bacterium grows. Because of this, it is possible that MVs entrap a proportion of these peptidoglycan hydrolyzing enzymes during MV formation[4]. Also it has been reported that, during their formation, MVs entrap periplasmic components; for *Pseudomonas aeruginosa* these include alkaline phosphatase, phospholipase C, proelastase, protease and peptidoglycan hydrolase[5-7]. Accordingly MVs are small bilayered particles into which degradative enzymes are concentrated[8].

MVs are capable of lysing a variety of gram-positive and gram-negative bacteria and the potency of lysis depends on the peptidoglycan chemotype of the attacked cell[4]. For gram-positive cells, MVs adhere to the cell wall, where they break up and digest the immediate underlying peptidoglycan. MVs attack on gram-negative bacteria in much different manner. Here, MVs adhere to the outer membrane and rapidly fuse into it[6] and the luminal

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contents (including peptidoglycan hydrolase) are released directly into the periplasmic space of the recipient cell. Inside the periplasmic space, peptidoglycan hydrolase can fully diffuse around the protoplast and hydrolyze the peptidoglycan layer at number of different sites so that multisite lysis can occur. MV-mediated lysis of bacteria occurs only when the recipient cells are under insufficient nutrient conditions[4].

In pathogenic gram-negative bacteria, it has been reported that virulence factors required for infection are encapsulated into MVs and released into the site of infection[9-13]. Also it has been reported that MVs of *Pseudomonas aeruginosa* were enriched with a particular variety of lypopolysaccharide from the cell surface[5]. Furthermore, discrete enzymes such as alkaline phosphatase, phospholipase C and protease were packaged into MVs and each of these components is important in the pathogenesis of *Pseudomonas* infections.

It is possible that MVs have a predatory role in the natural ecosystem in which they are released by the parent bacterium to lyse the surrounding cells, increasing availability of nutrients to the parent strain[8]. The predatory role of MVs against gram-negative and gram-positive bacteria can be increased by gentamicin treatment. The gentamicin-MVs (g-MVs) would possess higher lytic activity against hard-to-kill clinical isolates because of the entrapped gentamicin.

The present research was concerned with the study of outer membrane vesicles formation by different gram-negative bacteria and their bacteriolytic activity against a variety of gram-positive and gram-negative bacteria. The effect of the β-lactam antibiotic cefotaxime and the aminoglycoside antibiotic gentamicin on the formation, size and lytic activity of MVs formed by these bacteria was also investigated.

MATERIALS AND METHODS

Bacterial strains: *Enterobacter cloacae* NCTC10005, *Proteus vulgaris* 1753 and *Serratia marcescens* HIM 307-2 (clinical isolates with inducible chromosomal β-lactamase) were kindly provided by Professor H.H. Martin, Institute of Microbiology, TH Darmstadt, Germany. *Erwinia carotovora* NCPPB312 and *Erwinia carotovora* NCPPB671 (plant pathogens) were obtained from The National Collection of Plant Pathogenic Bacteria, Ministry of Agriculture, Fisheries and Food Plant Pathology Laboratory, Harpenden, UK. *Pseudomonas syringae* pv. *Syringae* Van Hall strain 347448 was obtained from the International Mycological Institute, Bakeham Lane, Egham, Surrey TW20 9TY, UK. *Bacillus cereus* 1080, *Bacillus subtilis* 1020, *E. coli* 1357 and *Pseudomonas solanacearum* B-3212 were obtained from the culture collection of the Microbiological Research Center (MIRCEN), Faculty of Agriculture, Ain-Shams University, Cairo, Egypt. *Citrobacter freundii* and *Klebsiella pneumoniae* (clinical isolates) were kindly obtained from Department of Microbiology, Faculty of Pharmacy, Tanta University, Tanta, Egypt

Antibiotics: Cefotaxime (T3A Pharme Group, Egypt) and gentamicin (Glaxo Wellcome, Egypt) were commercially available.

Determination of minimal inhibitory concentration (MICs): The MICs of cefotaxime and gentamicin for the tested bacteria were determined in nutrient broth (Oxoid, England) by two-fold serial dilution technique described by Deutshe Normen, DIN 58, 490, part 5 (1975), yielding a final inoculum of $5x10^5$ CFU mL^{-1} in sterile 6x100 mm capped tubes.

Exposure of MV-producing bacteria to cefotaxime and gentamicin: The exposure of the tested bacteria to cefotaxime and gentamicin to study their effects on MVs formation was performed according to the method described by Kadurugamuwa and Beveridge[5], after modifications. The tested organisms were grown to the mid log phase (OD_{600} = 0.4 to 0.5) in nutrient broth at agitation rate of 150 rpm. Cefotaxime and gentamicin were added to the cultures separately and the incubation of antibiotic-treated and untreated cultures was allowed to continue to the early stationary phase at reduced agitation rate of 100 rpm. The cell sediments were separated by centrifugation (REMI laboratory centrifuge R32A) at 3000 rpm for 20 min, washed twice with 50 mM potassium-sodium-phosphate buffer pH 7 and then resuspended in the same buffer and kept at 4°C until required.

Lytic activity of the MVs: The bacteriolytic activity of MVs produced by the investigated gram-negative bacteria against different gram-positive and gram-negative hosts was determined by plate assay technique[14]. Overnight cultures of the host bacteria were prepared in nutrient broth and the cells were harvested by centrifugation and washed twice by K-Na-phosphate buffer. 10^5 CFU of the washed cells suspended in 100 μL buffer were spread on the surface of agar plates (15 g agar/one liter of phosphate buffer). Cells of 0.25 mL of early stationary phase of antibiotic–treated and untreated MV-producing cultures prepared in nutrient broth were harvested and washed twice with buffer. The washed cells suspended in 100 μL buffer were loaded into wells previously made in agar

plates. The cultivated plates were incubated overnight at the temperature of the host bacteria and bacteriolysis of the host bacteria was seen as clear zones around the wells[14].

Transmission electron microscopy (TEM): Twenty microliter of cell suspension of the antibiotic-treated and untreated cultures was placed on carbon-coated nickel grids and stained with 2% aqueous solution of uranyl acetate for 15-20 sec. The stained grids were rinsed and examined with ZEISS EM10 transmission electron microscope operating under standard conditions[14].

RESULTS

The MICs of cefotaxime and gentamicin for *Citrobacter freundii, Enterobacter cloacae* NCTC10005, *Erwinia carotovora* NCPPB312, *Klebsiella pneumoniae, Proteus vulgaris* 1753 and *Serratia marcescens* HIM 307-2, were determined to select the concentration of each antibiotic suitable for treatment of MV-forming bacteria (Table 1). The concentrations of cefotaxime and gentamicin presented in Table 2 were found to be suitable and used for treatment of MV-forming bacteria without deleterious effects on their growth, where the antibiotics were added to the cultures at the mid log phase.

Bacteriolytic activity and electron microscopy: The results produced by the plate assay technique showed that different lytic potencies were obtained by the MVs produced by *Citrobacter freundii, Enterobacter cloacae* NCTC10005, *Erwinia carotovora* NCPPB312, *Klebsiella pneumoniae, Proteus vulgaris* 1753 and *Serratia marcescens* HIM 307-2 against different gram-positive and gram-negative host bacteria. Also the transmission electron microscopy revealed that the MVs produced by these bacteria varied in size and amounts.

MVs of *C. freundii*: As shown in Table 3, the MVs formed by *C. freundii* had lytic activities only against half of the host bacteria tested, where no killing effect was recorded against *B. subtilis* 1020, *E. cloacae* NCTC10005, *E. carotovora* NCPPB312, *E. carotovora* NCPPB671 and *P. solanacearum* B-3212. On the other hand, lytic activities expressed as clear zones (Fig. 1) were obtained against the rest of tabulated host bacteria. *C. freundii* cultures exposed to cefotaxime (8 μg mL^{-1}) or gentamicin (1 μg mL^{-1}) produced MVs (cefotaxime MVs or gentamicin MVs, respectively) with higher lytic activities against the susceptible hosts compared to untreated culture (natural MVs). The MVs produced by *C. freundii* under normal growth conditions had spherical shapes and size of 70 nm.

Table 1: Minimal inhibitory concentrations of cefotaxime and gentamicin for the tested MV-forming gram-negative bacteria

	MICs of antibiotics (μg mL^{-1})	
Bacteria strains	Cefotaxime	Gentamicin
Citrobacter freundii	4	0.5
Enterobacter cloacae NCTC10005	128	0.25
Erwinia carotovora NCPPB312	<0.0625	0.25
Klebsiella pneumoniae	4	0.5
Proteus vulgaris 1753	8	1
Serratia marcescens HIM 307-2	32	0.5

Table 2: Concentrations of cefotaxime and gentamicin used for treatment of MV-forming gram-negative bacteria

	Concentrations (μg mL^{-1})	
Bacteria strains	Cefotaxime	Gentamicin
Citrobacter freundii	8	1
Enterobacter cloacae NCTC10005	512	1
Erwinia carotovora NCPPB312	0.0625	0.75
Klebsiella pneumoniae	16	2
Proteus vulgaris 1753	32	4
Serratia marcescens HIM 307-2	64	2

Table 3: Lytic effects of MVs formed by *Citrobacter freundii* on different bacteria

	Clear zone (mm) caused by MVs of *C. freundii*		
Host bacteria	n-MVs	c-MVs	g-MVs
Bacillus cereus 1080	14.7±0.07	23.5±0.07	18.0±0.14
Bacillus subtilis 1020	0.0	0.0	0.0
Enterobacter cloacae NCTC 10005	0.0	0.0	0.0
Erwinia carotovora NCPPB 312	0.0	0.0	0.0
Erwinia carotovora NCPPB 671	0.0	0.0	0.0
Escherichia coli 1357	17.8±0.07	24.2±0.10	22.8±0.034
Proteus vulgaris 1753	8.65±0.23	22.0±0.12	23.1±0.12
Pseudomonas solanacearum B-3212	0.0	0.0	0.0
Pseudomonas syringae	18.55±0.007	26.0±0.10	21.5±0.35
Serratia marcescens HIM 307-2	19.3±0.058	19.5±0.12	23.3±0.06

n-MVs: Natural membrane vesicles, c-MVs: Cefotaxime membrane vesicles, g-MVs: Gentamicin membrane vesicles, ± :Standard error of means

Table 4: Lytic effects of MVs formed by *Enterobacter cloacae* NCTC10005 on different bacteria

	Clear zone (mm) caused by MVs of *E. cloacae* 10005		
Host bacteria	n-MVs	c-MVs	g-MVs
Bacillus cereus 1080	0.0	0.0	0.0
Bacillus subtilis 1020	15.4±0.06	0.0	20.5±0.20
Erwinia carotovora NCPPB312	17.3±0.10	0.0	26.0±0.10
Erwinia carotovora NCPPB671	0.0	0.0	18.5±0.05
Escherichia coli 1357	17.0±0.10	0.0	25.0±0.37
Proteus vulgaris 1753	18.1±0.14	0.0	22.6±0.06
Pseudomonas solanacearum B-3212	17.1±0.07	0.0	22.9±0.10
Pseudomonas syringae	17.6±0.06	0.0	20.6±0.28
Serratia marcescens HIM 307-2	0.0	0.0	0.0

Abbreviations are defined in footnote to Table 3

Table 5: Lytic effects of MVs formed by *Erwinia carotovora* NCPPB312 on different bacteria

Host bacteria	Clear zone (mm) caused by MVs of *E. carotovora* NCPPB312		
	n-MVs	c-MVs	g-MVs
Bacillus cereus 1080	18.0±0.14	20.5±0.07	20.0±0.14
Bacillus subtilis 1020	15.6±0.06	16.1±0.07	19.0±0.14
Enterobacter cloacae NCTC10005	19.5±0.07	20.2±0.11	21.8±0.04
Erwinia carotovora NCPPB671	0.0	23.6±0.11	21.0±0.11
Escherichia coli 1357	19.0±0.14	19.5±0.07	21.5±0.07
Proteus vulgaris 1753	0.0	20.5±0.07	22.2±0.24
Pseudomonas solanacearum B-3212	16.7±0.11	16.5±0.03	19.0±0.40
Pseudomonas syringae	18.5±0.07	18.8±0.11	21.6±0.03
Serratia marcescens HIM 307-2	0.0	21.0±0.14	20.5±0.07

Abbreviations are defined in footnote to Table 3

Table 6: Lytic effects of MVs formed by *Klebsiella pneumoniae* on different bacteria

Host bacteria	Clear zone (mm) caused by MVs of *K. pneumoniae*		
	n-MVs	c-MVs	g-MVs
Bacillus cereus 1080	0.0	35.0 ± 0.5	0.0
Bacillus subtilis 1020	0.0	33.2 ± 0.04	0.0
Enterobacter cloacae NCTC10005	15.5±0.07	30.0±0.12	19.5±0.07
Erwinia carotovora NCPPB312	0.0	31.5±0.2	0.0
Erwinia carotovora NCPPB671	0.0	29.5±0.07	19.3±0.15
Escherichia coli 1357	19.15±0.12	31.0±0.10	23.7±0.15
Proteus vulgaris 1753	19.97±0.20	20.32±0.15	22.5±0.07
Pseudomonas solanacearum B-3212	0.0	29.0±0.30	0.0
Pseudomonas syringae	0.0	34.0±0.14	22.0±0.10
Serratia marcescens HIM 307-2	19.55±0.01	32.5±0.20	23.3±0.08

Abbreviations are defined in footnote to Table 3

Table 7: Lytic effects of MVs formed by *Proteus vulgaris* 1753 on different bacteria

Host bacteria	Clear zone (mm) caused by MVs of *P. vulgaris* 1753		
	n-MVs	c-MVs	g-MVs
Bacillus cereus 1080	15.8±0.03	20.3±0.06	27.0±0.15
Bacillus subtilis 1020	16.5±0.07	22.5±0.07	24.0±0.14
Enterobacter cloacae NCTC10005	18.0±0.14	27.5±0.07	26.1±0.10
Erwinia carotovora NCPPB312	15.6±0.06	18.4±0.10	17.6±0.06
Erwinia carotovora NCPPB671	0.0	20.0±0.07	23.5±0.07
Escherichia coli 1357	15.8±0.03	25.0±0.14	23.4±0.07
Pseudomonas solanacearum B-3212	16.5±0.07	23.5±0.07	23.8±0.01
Pseudomonas syringae	0.0	0.0	0.0
Serratia marcescens HIM 307-2	16.5±0.07	30.0±0.14	20.75±0.03

Abbreviations are defined in footnote to Table 3

MVs of *Enterobacter cloacae* NCTC10005: *E. cloacae* NCTC10005 produced natural MVs (n-MVs) under normal growth conditions and gentamicin MVs (g-MVs) after exposure to 1 μg mL^{-1} of gentamicin. No MVs could be produced by this bacterium after exposure to the β-lactam antibiotic, cefotaxime (Fig. 2b) The n-MVs formed by

Table 8: Lytic effects of MVs formed by *Serratia marcescens* HIM 307-2 on different bacteria

Host bacteria	Clear zone (mm) caused by MVs of *S. marcescens* HIM 307-2		
	n-MVs	c-MVs	g-MVs
Bacillus cereus 1080	19.0±0.14	19.5±0.07	23.9±0.11
Bacillus subtilis 1020	23.0±0.10	25.5±0.53	27.1±0.08
Enterobacter cloacae NCTC10005	0.0	0.0	0.0
Erwinia carotovora NCPPB312	18.3±0.07	17.3±0.06	23.1±0.12
Erwinia carotovora NCPPB671	0.0	0.0	21.5±0.20
Escherichia coli 1357	19.0±0.28	18.5±0.07	24.25±0.04
Proteus vulgaris 1753	16.5±0.07	20.1±0.36	21.0±0.10
Pseudomonas solanacearum B-3212	31.3±0.30	22.8±0.14	35.5±0.07
Pseudomonas syringae	30.2±0.12	24.3±0.15	32.1±0.10

Abbreviations are defined in footnote to Table 3

Fig. 1: Lytic effect of MVs of *C. freundii* on *Serratia marcescens* HIM 307-2, expressed as diameter of clear zone produced on agar plate

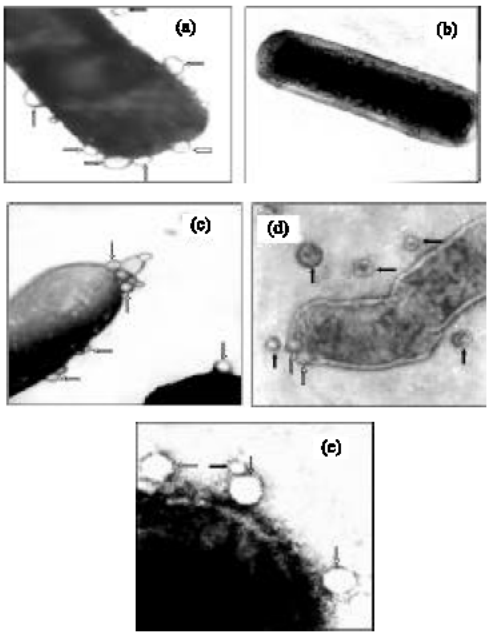

Fig. 2: Transmission electron micrograph of (a), n-MVs and (b) cefotaxime-treated cells of *E. cloacae* NCTC10005 (no MVs) (c) n-MVs and (d) c-MVs of *K. pneumoniae* and (e) n-MVs of *P. vulgaris* 1753 (open arrows for surface MVs and solid arrows for free MVs)

E. cloacae NCTC10005 showed bacteriolytic activities against *B. subtilis* 1020, *Erwinia carotovora* NCPPB312, *E. coli* 1357, *Proteus vulgaris* 1753, *Pseudomonas solanacearum* B-3212 and *P. syringae*, recording clear zones around 17 mm (Table 4). The g-MVs produced by this bacterium had a higher lytic activity against the susceptible hosts. The transmission electron micrograph of *E. cloacae* NCTC10005 washed cells showed that the MVs produced under normal growth conditions had spherical forms and size of 105 nm (Fig. 2a).

MVs of *Erwinia carotovora* NCPPB312: *E. carotovora* NCPPB312 is a phytopathogen, it produced MVs under normal growth conditions (n-MVs) and after exposure to 0.0625 μg mL^{-1} of cefotaxime (c-MVs) or 0.75 μg mL^{-1} of gentamicin (g-MVs). The n-MVs produced by this bacterium exhibited killing effects against the gram-positive and gram-negative hosts except *E. carotovora* NCPPB671, *Proteus vulgaris* 1753 and *S. marcescens* HIM 307-2. On the other hand, c-MVs and g-MVs had higher lytic activities against all of the host bacteria compared to those of n-MVs (Table 5). The n-MVs formed by this organism had spherical forms and size of 55 nm.

MVs of *Klebsiella pneumoniae*: Table 6 showed that both n-MVs and g-MVs produced by this organism showed no lytic activity against *B. cereus* 1080, *B. subtilis* 1020, *E. carotovora* NCPPB312 and *P. solanacearum* B-3212. High killing effects against all of the host bacteria were recorded by c-MVs when the culture of the producing organism exposed to 16 μg mL^{-1} of cefotaxime at the mid log phase. The n-MVs of *K. pneumoniae* had a size of 35 nm while cefotaxime induced the formation of larger MVs with size of 200 nm (Fig. 2c and d).

MVs of *Proteus vulgaris* 1753: The MVs formed by *P. vulgaris* 1753 showed killing activities against most of the host bacteria tested (Table 7). The n-MVs formed by this organism had a bacteriolytic activity against the all tested bacteria except *E. carotovora* NCPPB671 and *P. syringae*. Although the n-MVs had no effect on the growth of *E. carotovora* NCPPB671, c-MVs and g-MVs produced by the same bacterium were active against the same host. Both n-MVs, c- MVs and g-MVs recorded no deleterious effect on the growth of *P. syringae*. The n-MVs formed by *P. vulgaris* 1753 possessed spherical forms and size of 125 nm under the experimental conditions used (Fig. 2e).

MVs of *Serratia marcescens* HIM 307-2: The n-MVs formed by *S. marcescens* HIM 307-2, lysed all gram-positive and gram-negative host bacteria except *E. carotovora* NCPPB671 and *E. cloacae* NCTC10005 (Table 8). The g-MVs showed higher killing effects against the susceptible hosts compared to those caused by n-MVs or c-MVs. *S. marcescens* HIM 307-2 formed spherical MVs (30 nm in size) under normal growth conditions and after exposure to 2 μg mL^{-1} of gentamicin (36 nm) and tubular and spherical MVs (63 nm) after exposure to 64 μg mL^{-1} of cefotaxime.

DISCUSSION

Many gram-negative bacteria produce external MVs during the normal growth. The release of these vesicles from the whole cell depends on the bacterial strains as well as the nutritional conditions[5,11,13,15-20]. All of the six gram-negative bacteria used in this study produced MVs when grown in nutrient broth. The MVs produced by the test bacteria, *Citrobacter freundii, Enterobacter cloacae* NCTC10005, *Erwinia carotovora* NCPPB312, *Klebsiella pneumoniae, Proteus vulgaris* 1753 and *Serratia marcescens* HIM 307-2 showed different lytic activities against a variety of gram-positive and gram-negative host bacteria (clinical isolates and plant pathogens). These MVs might contain hydrolytic enzymes that able to hydrolyze the peptidoglycan of the cell of the host bacteria. MV-mediated lysis of bacteria does not readily occur if the recipient cells are actively growing and dividing; it occurs only when they are under the constraints of poor nutrition[4]. Because poor growth conditions are required for efficient killing of other bacteria by MVs, we used in this study agar in phosphate buffer (for plate assay) to determine the bacteriolytic activity of MVs produced by the test gram-negatives against different recipients. The n-MVs produced by all of the gram-negative bacteria used in this study showed no lytic activity against *Erwinia carotovora* NCPPB671. This might be explained because the n-MVs produced by the donor bacteria might not be enough to kill this host and/or the peptidoglycan chemotype of the recipient bacterium might be completely different from those of the donor bacteria[4]. Almost all of the test MV-producing bacteria except *E. cloacae* NCTC10005 produced MVs with higher lytic activities against most of the recipient bacteria after exposure to cefotaxime or gentamicin. The higher lytic activities of the c-MVs and g-MVs might be due to the formation of larger MVs filled with degradative enzymes and cefotaxime or gentamicin which might act synergistically with the enzymes enclosed to lyse hard-to-kill hosts. In the case of *E. cloacae* NCTC10005, the exposure to the β-lactam antibiotic cefotaxime inhibited the formation of MVs by this bacterium. The inhibition of

MVs by *E. cloacae* NCTC10005 treated with cefotaxime might be explained because cefotaxime might be able to bind and inactivate the penicillin-binding-proteins (PBPs) present on the outer surface of the bacterial cell membrane which have a role in MVs formation[5,21-25]. Similar conclusion was reported by Azab[14] using *E. herbicola* 48 exposed to the β-lactam antibiotic cephradine. Because the amounts and the molecular weigh of PBPs differ from bacterium to other[26] and also the binding affinity of the β-lactam antibiotics (specially to high molecular weight PBPs) differ from organism[27] to other, the effect of cefotaxime on MVs formation by the test bacteria was different. In *K. pneumoniae*, cefotaxime induced the formation of the largest (200 nm) MVs compared to all antibiotic-treated and untreated donor bacteria. This may explain the highest lytic power of c-MVs produced by *K. pneumoniae*. The Mvs produced by *E. carotovora* NCPPB312 and *P. vulgaris* 1753 (under different growth conditions) exhibited high lytic potencies and the widest killing spectra while MVs produced by *C. freundii* showed the least killing spectrum.

Concerning the shape of the formed MVs, all of the test gram-negative bacteria produced spherical MVs under normal growth conditions, but in *S. marcescens* HIM 307-2, cefotaxime induced the formation of larger tubular MVs. Similar observation was reported by using *Erwinia herbicola* 48 exposed to gentamicin[14].

The sizes of MVs formed by all of the gram-negative bacteria used in this study, ranged between 30 and 200 nm. Both cefotaxime and gentamicin induced the formation of larger MVs in all producing organisms, giving rise 5 times the size of untreated in *K. pneumoniae* treated with cefotaxime.

Finally we could conclude that all of the gram-negative bacteria used (clinical isolate and plant pathogens), produced MVs under normal growth conditions. The lytic power and the killing spectrum of the MVs might depend on the producing strain, peptidoglycan chemotype of the recipient bacteria and the size and amount of the MVs produced. In most producing strains, cefotaxime and gentamicin induced the formation of MVs larger in size and had a higher killing power. In a medical and biocontrol contexts, the predatory role of MVs could be increased by cefotaxime or gentamicin treatment. The c-MVs and g-MVs would have more killing power against hard-to-kill pathogens because the associated antibiotics entrapped in the MVs would be delivered into the periplasm of the host cells together with the degradative enzymes. Also Beveridge[4] suggested that MVs could be used to deliver drugs to a number of different prokaryotic and eukaryotic systems. For the natural MVs, there is another possible medical use as vaccine agents. During their release from certain bacterium, MVs entrap some periplasmic component including the identity of the donor bacterium. Lipopolysaccharide (LPS) is LPS serotype and strain specific and the same is true for outer membrane proteins. So, MVs are strong antigenic structures and could have the ability to enhance an immune response.

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