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Dissemination of the *bla*$_{CTX-M-15}$ gene among Enterobacteriaceae via outer membrane vesicles

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Background: Bacterial outer membrane vesicles (OMVs) are an emerging source of antibiotic resistance transfer but their role in the spread of the *bla*$_{CTX-M-15}$ gene encoding the most frequent CTX-M ESBL in Enterobacteriaceae is unknown.

Objectives: To determine the presence of *bla*$_{CTX-M-15}$ and other antibiotic resistance genes in OMVs of the CTX-M-15-producing MDR *Escherichia coli* O104:H4 outbreak strain and the ability of these OMVs to spread these genes among Enterobacteriaceae under different conditions.

Methods: OMV-borne antibiotic resistance genes were detected by PCR; OMV-mediated transfer of *bla*$_{CTX-M-15}$ and the associated *bla*$_{TEM-1}$ was quantified under laboratory conditions, simulated intraintestinal conditions and under ciprofloxacin stress; resistance to antibiotics and the ESBL phenotype were determined by the CLSI disc diffusion methods and the presence of pESBL by plasmid profiling and Southern blot hybridization.

Results: *E. coli* O104:H4 OMVs carried *bla*$_{CTX-M-15}$ and *bla*$_{TEM-1}$ located on the pESBL plasmid, but not chromosomal antibiotic resistance genes. The OMVs transferred *bla*$_{CTX-M-15}$, *bla*$_{TEM-1}$ and the associated pESBL into Enterobacteriaceae of different species. The frequencies of the OMV-mediated transfer were significantly increased under simulated intraintestinal conditions and under ciprofloxacin stress when compared with laboratory conditions. The 'vesiculants' (i.e. recipients that received the *bla*$_{CTX-M-15}$- and *bla*$_{TEM-1}$-harbouring pESBL via OMVs) acquired resistance to cefotaxime, ceftazidime and cefpodoxime and expressed the ESBL phenotype. They were able to further spread pESBL and the *bla*$_{CTX-M-15}$ and *bla*$_{TEM-1}$ genes via OMVs.

Conclusions: OMVs are efficient vehicles for dissemination of the *bla*$_{CTX-M-15}$ gene among Enterobacteriaceae and may contribute to *bla*$_{CTX-M-15}$ transfer in the human intestine.

Introduction

The global spread of Enterobacteriaceae producing ESBLs of CTX-M type in the last 20 years represents a serious medical and public health problem.[1–5] Increasing production of CTX-M ESBLs, the most prevalent of which are CTX-M-15 and CTX-M-14,[3–6] has been mainly observed in Enterobacteriaceae that cause extraintestinal infections, particularly in *Escherichia coli* and *Klebsiella pneumoniae*,[2–6] but also in intestinal pathogens, including diarrhoeagenic *E. coli*,[7–10] salmonellae[11–14] and shigellae.[11,13,15–19] The major reasons for the rapid global dissemination of CTX-M ESBLs include: (i) predominant localization of CTX-M-encoding genes on conjugative plasmids, which facilitate their intraspecies, interspecies and intergenus horizontal transfer;[3,4,20,21] (ii) genetic linkage of these genes to ISs, transposons or integrons, which promote their genomic mobilization;[3,4] and (iii) their frequent association with worldwide-distributed highly virulent clones (high-risk clones) of *E. coli* and *K. pneumoniae* that mostly carry *bla*$_{CTX-M-15}$.[4,5,22–24]

The horizontal transfer of *bla*$_{CTX-M}$ and other ESBL genes via conjugative plasmids is currently the best understood mechanism of their global spread.[3,4,20,21] However, novel mechanisms of antibiotic resistance gene (ARG) transfer have been emerging. Specifically, outer membrane vesicles (OMVs), multifunctional nanoparticles released from the bacterial outer membrane that contain numerous biomolecules including DNA,[25,26] can mediate horizontal transfer of ARGs.[27–33] This ability has been demonstrated for OMVs from *Acinetobacter* spp.,[29,30,32] *Neisseria gonorrhoeae*,[27] *E. coli*[28] and *Porphyromonas gingivalis*[31] and involved genes encoding resistance to penicillins,[27,28,30]

carbapenems (bla_{OXA-24} and bla_{NDM-1}),[29,32] fluoroquinolones [*aac(6′)-Ib-cr*)][32] and erythromycin (*erm*).[31] However, the role of OMVs in the spread of bla_{CTX-M} genes remains unknown.

In 2011, the CTX-M-15-producing and MDR *E. coli* O104:H4 strain caused a large outbreak of diarrhoea complicated by haemolytic uraemic syndrome (HUS), which affected almost 4000 people and caused 54 deaths.[34,35] The outbreak strain was a hybrid of Shiga toxin-producing and enteroaggregative *E. coli*,[36–39] whose $bla_{CTX-M-15}$ gene was located on an ~90 kb plasmid (pESBL), which also carried bla_{TEM-1}, whereas the other ARGs [*dfrA7*, *sul2*, *strA*, *strB* and *tet*(A)] were on the chromosome.[37–39] We have previously demonstrated that the outbreak strain released OMVs, which contained the stx_{2a} gene encoding Shiga toxin 2a,[40] the major virulence factor of this strain.[36,41] This prompted us to investigate, in this study, OMVs from the outbreak strain for the presence of $bla_{CTX-M-15}$ and other ARGs of this pathogen and for their ability to disseminate these genes among Enterobacteriaceae. We also determined the molecular basis of OMV-mediated $bla_{CTX-M-15}$ transfer and the persistence of this transfer in 'vesiculants', i.e. recipients that acquired $bla_{CTX-M-15}$ via OMVs (the term was introduced by Fulsundar *et al.*[30]). Finally, we explored the influence of simulated human intraintestinal conditions and antibiotic stress on the extent of OMV-mediated $bla_{CTX-M-15}$ transfer.

Materials and methods

Bacterial strains

E. coli O104:H4 strain C227-11ϕcu (used as a primary OMV donor) is a derivative of the *E. coli* O104:H4 outbreak strain, cured of the stx_{2a}-harbouring bacteriophage.[40,41] Like the outbreak strain,[36,42] strain C227-11ϕcu displays the ESBL phenotype and is also resistant to trimethoprim/sulfamethoxazole, streptomycin, tetracycline and nalidixic acid (Table 1). The $bla_{CTX-M-15}$ gene is located, together with bla_{TEM-1}, on pESBL[38] and *dfrA7*, *sul2*, *strA*, *strB* and *tet*(A) on the chromosome.[39] Clinical Enterobacteriaceae isolates used as recipients for C227-11ϕcu OMV-associated ARGs are listed in Table 1. *E. coli* K-12 C600 was a positive control recipient.[28,30]

Antibiotic susceptibility testing

Susceptibility to ampicillin, cefotaxime, ceftazidime, cefpodoxime, meropenem, imipenem, trimethoprim/sulfamethoxazole, ciprofloxacin, gentamicin, kanamycin, azithromycin, tigecycline, tetracycline, streptomycin

Table 1. OMV donor and recipients for OMVs from *E. coli* O104:H4 strain C227-11ϕcu

Strain designation or no.[a]	Species (pathotype, serotype)[b]	Origin	Diagnosis[c]	Resistance[d]	PCR result	
					$bla_{CTX-M-15}$[e]	bla_{TEM-1}[e]
C227-11ϕcu	*E. coli* O104:H4 outbreak strain, stx_{2a} phage-cured derivative	stool	bloody diarrhoea	AMP, CTX, CAZ, CPD[f], STR, NAL, TET, SXT	+	+
C600	*E. coli* K-12	laboratory strain	NA	none	–	–
11/08	*E. coli* non-pathogenic	stool	healthy	none	–	–
29/10	*E. coli* (EPEC, O26: H11)	stool	diarrhoea	AMP, GEN	–	+
125/12	*E. coli* (STEC, O26:H11)	stool	HUS	none	–	–
455/13	*E. coli* (EAEC, O111:H21)	stool	diarrhoea	none	–	–
532/09	*E. coli* (ExPEC, O6:H1)	urine	UTI	none	–	–
326/11	*E. coli* (ExPEC, O8:H$^-$)	blood	sepsis	AMP, CIP	–	+
17/08	*Salmonella enterica* serovar Enteritidis	stool	diarrhoea	AMP, CIP	–	+
85/11	*Salmonella enterica* serovar Typhimurium	stool	diarrhoea	none	–	–
116/07	*Shigella sonnei*	stool	diarrhoea	AMP	–	+
179/02	*Shigella flexneri*	stool	diarrhoea	SXT	–	–
201/10	*K. pneumoniae*	blood	sepsis	AMP, CIP	–	–
126/12	*K. pneumoniae*	urine	UTI	AMP	–	–
181/09	*Enterobacter cloacae*	blood	sepsis	AMP	–	–
245/12	*E. cloacae*	urine	UTI	AMP, GEN, KAN	–	+
109/13	*Proteus mirabilis*	urine	UTI	none	–	–

AMP, ampicillin; CIP, ciprofloxacin; GEN, gentamicin; KAN, kanamycin; SXT, trimethoprim/sulfamethoxazole; NAL, nalidixic acid; STR, streptomycin; CAZ, ceftazidime; CPD, cefpodoxime; CTX, cefotaxime; TET, tetracycline.
[a]The two digits after the solidus in strain numbers indicate the year of isolation.
[b]EPEC, enteropathogenic *E. coli*; STEC, Shiga toxin-producing *E. coli*; EAEC, enteroaggregative *E. coli*; ExPEC, extraintestinal pathogenic *E. coli*.
[c]UTI, urinary tract infection; NA, not applicable.
[d]Determined by the disc diffusion method. 'None' indicates susceptible to all 15 antimicrobials tested (see Methods).
[e]+, amplicon present; –, amplicon absent.
[f]ESBL phenotype was confirmed by the CLSI double disc diffusion method (see Methods).

and nalidixic acid was tested using the disc diffusion method according to CLSI procedures and criteria.[43] ESBL phenotype was confirmed using the CLSI double disc diffusion method,[43] performed with ceftazidime and ceftazidime/clavulanate, and cefotaxime and cefotaxime/clavulanate discs, respectively (ESBL detection disc set; Mast Group Ltd.). According to CLSI criteria, the results were considered positive if the diameter of the inhibitory zone increased by ≥5 mm in the presence of clavulanate compared with that in the absence of clavulanate.

OMV isolation and treatments

OMVs were isolated, as previously described,[40,44,45] from stationary phase (16 h) cultures (Figure S1, available as Supplementary data at *JAC* Online). To prepare OMVs under standard laboratory conditions (OMVs-$_{LB}$), strains were grown in LB broth, cultures were centrifuged and supernatants were sterile-filtered (0.22 μm Stericup filters; Merck). OMVs were collected by ultracentrifugation (235 000 **g**, 2 h, 4°C) and resuspended in PBS (pH 7.2). To produce OMVs under simulated intraintestinal conditions, strain C227-11ϕcu was cultured in simulated ileal environment medium (SIEM) or simulated colonic environment medium (SCEM);[44] OMVs (OMVs-$_{SIEM}$ and OMVs-$_{SCEM}$, respectively) were isolated as above. To produce OMVs under antibiotic stress, strain C227-11ϕcu was grown in LB broth to an OD_{600} of ∼0.5, then ciprofloxacin (Sigma–Aldrich) (0.0625 mg/L, corresponding to ¼ MIC)[45] was added and culture was continued until 16 h, when OMVs (OMVs-$_{CIP}$) were isolated. All OMV preparations were treated with 100 μg/mL proteinase K (PK) to digest phage coats (if present) and 100 ng/mL DNase (both Sigma–Aldrich) to remove extravesicular DNA.[30,40] This DNase concentration degraded all C227-11ϕcu OMV-associated extravesicular DNA tested in our previous study.[40] The mixtures were incubated at 37°C for 20 min followed by DNase inactivation (65°C, 10 min).[30] Treated OMVs were sterilized through 0.22 μm filters and sterilities were verified by culturing aliquots on blood agar.

OMV characterization

PK and DNase (PK/DNase)-treated OMVs prepared under different conditions were characterized as follows. Protein concentrations were determined with Roti-Nanoquant (Roth). To determine DNA concentrations, OMVs were lysed (0.125% Triton-X-100, 30 min, 37°C), intravesicular DNA was purified (High Pure PCR Product Purification Kit; Roche) and quantified (Quant-iT-PicoGreen dsDNA Assay Kit; Life Technologies).[40] OMV sizes and counts were determined by nanoparticle tracking analysis (NTA) with a NanoSight LM-10 instrument (NanoSight).[44] OMV protein profiles were visualized by OMV gel electrophoresis (5 μg protein/lane) and silver staining.[40] Transmission electron microscopy (TEM) of negatively stained OMVs[32] was performed with an HT7800 electron microscope (Hitachi).

Detection of ARGs

PCRs were performed with the primers listed in Table S1 in 25 μL volumes. For OMV PCRs, 2.5 μL of PK/DNase-treated OMVs, either intact or lysed (0.125% Triton-X-100) after the treatment, or purified OMV DNA served as templates. C227-11ϕcu and *E. coli* C600 total DNA were positive and negative controls, respectively. Detection of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1}$ in recipients and vesiculants was performed with heat-extracted DNA.

OMV-mediated $bla_{CTX\text{-}M\text{-}15}$ transfer under laboratory conditions

OMV-mediated $bla_{CTX\text{-}M\text{-}15}$ transfer under standard laboratory conditions was performed, as previously described[28–30,32] with slight modifications. Recipients were grown in LB broth (37°C, 180 rpm) to an OD_{600} of 0.4, centrifuged and resuspended in LB broth to ∼10^7 cfu/mL. *E. coli* C600 suspension (100 μL) was mixed with PK/DNase-treated C227-11ϕcu OMVs-$_{LB}$ containing 1, 5, 10 or 50 ng of DNA [corresponding to ∼4, 21, 42 or 209 μg of OMV protein, respectively (Table S2)]; 100 μL of clinical Enterobacteriaceae recipients were mixed with C227-11ϕcu OMVs-$_{LB}$ containing 10 ng of DNA (∼42 μg protein). LB broth was added up to 1 mL and the mixtures were incubated at 37°C in the presence of DNase (final concentration of 100 ng/mL) for 3 h stationary and 3 h at 180 rpm. After adding LB broth up to 10 mL, incubation was continued overnight (37°C, 180 rpm). Bacteria were centrifuged, resuspended in 1 mL of LB broth and 10-fold dilutions were incubated overnight on LB agar (to determine total bacterial counts) and on ESBL agar (chromID ESBL; bioMérieux) to select $bla_{CTX\text{-}M\text{-}15}$-carrying vesiculants. The frequency of OMV-mediated $bla_{CTX\text{-}M\text{-}15}$ transfer was calculated as the number of vesiculants (cfu/mL on ESBL agar) in the total bacterial count (cfu/mL on LB agar). Ten randomly selected vesiculants of each recipient were confirmed for $bla_{CTX\text{-}M\text{-}15}$ by PCR and for resistance to cefotaxime, ceftazidime and cefpodoxime and ESBL phenotype as above. In $bla_{TEM\text{-}1}$-negative recipients, the vesiculants were also PCR-tested for $bla_{TEM\text{-}1}$. In control experiments, recipients were exposed to: (i) OMV buffer; (ii) OMVs (10 ng of DNA), which had been lysed with 0.125% Triton-X-100 to release intravesicular DNA; and (iii) C227-11ϕcu plasmid DNA (10 ng) isolated with the Plasmid Mini Kit (QIAGEN).

To determine whether the vesiculants that acquired $bla_{CTX\text{-}M\text{-}15}$ via C227-11ϕcu OMVs (termed 'first-generation vesiculants'; 1st GVs) could further disseminate $bla_{CTX\text{-}M\text{-}15}$ via OMVs, OMVs were isolated from three randomly selected 1st GVs of each recipient, PK/DNase-treated as above, PCR-confirmed for $bla_{CTX\text{-}M\text{-}15}$ and used [10 ng of DNA corresponding to ∼29–58 μg of OMV protein (Table S2)] in transfer experiments with additional clinical Enterobacteriaceae recipients; the resulting 'second-generation vesiculants' (2nd GVs) were selected on ESBL agar. The frequency of $bla_{CTX\text{-}M\text{-}15}$ transfer was calculated and the ESBL phenotype in 2nd GVs was confirmed as above.

OMV-mediated $bla_{CTX\text{-}M\text{-}15}$ transfer under simulated intraintestinal conditions

Recipients were grown in SIEM or SCEM to an OD_{600} of 0.4 and 100 μL of each suspension (∼10^6 cfu/mL) was mixed with PK/DNase-treated C227-11ϕcu OMVs-$_{SIEM}$ or OMVs-$_{SCEM}$ containing 10 ng of OMV-DNA [corresponding to ∼13 or ∼14 μg of OMV protein, respectively (Table S2)]. SIEM or SCEM was added up to 1 mL and the mixtures were incubated in the presence of DNase (100 ng/mL) for 3 h stationary, 3 h at 180 rpm and, after adding SIEM or SCEM, up to 10 mL, overnight (37°C, 180 rpm). Cultures were centrifuged, total bacterial counts and numbers of $bla_{CTX\text{-}M\text{-}15}$-carrying vesiculants (cfu/mL) were determined on LB agar and ESBL agar, respectively, and the frequencies of OMV-mediated $bla_{CTX\text{-}M\text{-}15}$ transfer were calculated as above. $bla_{CTX\text{-}M\text{-}15}$ and ESBL phenotype were confirmed as described above in 10 randomly selected vesiculants.

$bla_{CTX\text{-}M\text{-}15}$ transfer via OMVs-$_{CIP}$

The $bla_{CTX\text{-}M\text{-}15}$ transfer via PK/DNase-treated C227-11ϕcu OMVs-$_{CIP}$ was performed in LB broth, SIEM and SCEM, using 10 ng of OMV-DNA [corresponding to ∼14 μg of OMV protein (Table S2)] and recipients and procedures described above.

Plasmid profiling and Southern blot hybridization

Plasmid profiles were determined as previously described.[46] For Southern blot hybridization, plasmids were transferred to a nylon membrane and hybridized (DIG DNA Labelling and Detection Kit; Roche) with digoxigenin-11-dUTP-labelled $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1}$ probes, generated with the primers in Table S1 and a PCR DIG Probe Synthesis Kit (Roche).

Results

Presence of ARGs in E. coli O104:H4 OMVs

DNA associated with OMVs from *E. coli* O104:H4 strain C227-11ϕcu contained the $bla_{CTX-M-15}$ and bla_{TEM-1} genes located on pESBL[38] (for pESBL map see Figure S2) but not the chromosomal genes *dfrA7*, *sul2*, *strA*, *strB* or *tet*(A) (Table S3). The DNA encoding $bla_{CTX-M-15}$ and bla_{TEM-1} was packaged inside vesicles as demonstrated by: (i) its resistance to DNase in intact OMVs and sensitivity to DNase in lysed OMVs; and (ii) its detection in lysed OMVs not treated with DNase and in purified intravesicular DNA (Table S3).

E. coli O104:H4 OMVs transfer $bla_{CTX-M-15}$ and bla_{TEM-1} into various Enterobacteriaceae

To find out whether C227-11ϕcu OMVs could transfer the $bla_{CTX-M-15}$ gene and to determine the OMV-DNA amount required, *E. coli* strain C600 was incubated with increasing doses of PK/DNase-treated C227-11ϕcu OMVs-$_{LB}$ and the frequencies of $bla_{CTX-M-15}$ transfer were calculated by dividing the number of vesiculants (cfu/mL on ESBL agar) by the total bacterial count (cfu/mL on LB agar). The transfer frequencies were $(7.4\pm3.1)\times10^{-9}$, $(1.2\pm0.8)\times10^{-8}$, $(9.8\pm4.9)\times10^{-8}$ and $(7.6\pm4.5)\times10^{-8}$ for OMVs containing 1, 5, 10 and 50 ng of DNA, respectively [corresponding to ∼4, 21, 42 and 209 μg of OMV protein, respectively (Table S2)], demonstrating a dose-dependent transfer increase between 1 and 10 ng of OMV-DNA, but no further increase with 50 ng. We therefore used 10 ng of OMV-DNA (∼42 μg of OMV protein) in all further experiments. The number of OMVs-$_{LB}$ required to transfer 10 ng of DNA was 6.9×10^{9}, which represents 8.6% of the total OMVs-$_{LB}$ population (particles/mL) (Table S4). No vesiculants were obtained with lysed OMVs, OMV buffer or C227-11ϕcu plasmid DNA, demonstrating that intact OMVs are required for the $bla_{CTX-M-15}$ transfer.

Next, we determined the ability of PK/DNase-treated C227-11ϕcu OMVs-$_{LB}$ to transfer $bla_{CTX-M-15}$ into clinical Enterobacteriaceae isolates. Vesiculants were obtained with recipients of all seven species used, including a non-pathogenic *E. coli* and several extraintestinal and intestinal pathogens; the frequencies of the $bla_{CTX-M-15}$ transfer ranged from $(7.9\pm4.7)\times10^{-8}$ to $(2.8\pm2.1)\times10^{-7}$ (Figure 1a and Table S5). The presence of $bla_{CTX-M-15}$ was confirmed in all 10 randomly selected vesiculants of each recipient (Table S5). All these vesiculants acquired resistance to cefotaxime, ceftazidime and cefpodoxime, to which the recipients were susceptible, and expressed the ESBL phenotype (Table S5). Altogether, these experiments demonstrated that *E. coli* O104:H4 OMVs transfer $bla_{CTX-M-15}$ into Enterobacteriaceae of different species and that the gene is expressed and encodes biologically active CTX-M-15 ESBL in the vesiculants. Importantly, vesiculants of all 11 bla_{TEM-1}-lacking recipients (Table 1) acquired, besides $bla_{CTX-M-15}$, bla_{TEM-1} also (Table S5), suggesting that C227-11ϕcu OMVs transfer the $bla_{CTX-M-15}$- and bla_{TEM-1}-carrying pESBL plasmid.

E. coli O104:H4 OMVs transfer pESBL into vesiculants

To determine whether C227-11ϕcu OMVs transfer pESBL, we compared plasmid profiles of the C227-11ϕcu donor, those 11 bla_{TEM-1}-lacking recipients and the respective vesiculants and identified pESBL by hybridization with the $bla_{CTX-M-15}$ and bla_{TEM-1} probes. All vesiculants acquired a plasmid of ∼90 kb, which was present in the C227-11ϕcu OMV donor, but absent in the recipients (Table S6 and examples in Figure 2). This plasmid hybridized with both the $bla_{CTX-M-15}$ and bla_{TEM-1} probes (Table S6 and Figure 2) corroborating its identity as pESBL. No such plasmid was acquired when the recipients were incubated with lysed instead of intact C227-11ϕcu OMVs or C227-11ϕcu plasmid DNA (Table S6). These experiments demonstrated that *E. coli* O104:H4 OMVs can spread pESBL among Enterobacteriaceae.

OMVs from vesiculants further disseminate $bla_{CTX-M-15}$, bla_{TEM-1} and the associated pESBL

To explore the abilities of vesiculants that acquired $bla_{CTX-M-15}$ via C227-11ϕcu OMVs (1st GVs) to further disseminate the gene via OMVs, we used OMVs from the 1st GVs in transfer experiments with additional Enterobacteriaceae recipients (Table S7). OMVs from each 1st GV transferred $bla_{CTX-M-15}$ into each recipient, as demonstrated by acquisition of $bla_{CTX-M-15}$, resistance to cefotaxime, ceftazidime and cefpodoxime and expression of an ESBL phenotype by the resulting 2nd GVs (Table S8). The frequencies of the $bla_{CTX-M-15}$ transfer into the 2nd GVs via the 1st GV OMVs ranged from $(6.6\pm4.1)\times10^{-8}$ to $(3.4\pm2.2)\times10^{-7}$ (Table S9) and did not substantially differ with regard to the species of OMV donors and recipients. Notably, OMVs from the 1st GVs, in which we showed pESBL acquisition via C227-11ϕcu OMVs (Table S6 and Figure 2), transferred both $bla_{CTX-M-15}$ and bla_{TEM-1} into the 2nd GVs (Table S10), suggesting OMV-mediated transfer of pESBL from the 1st GVs to the 2nd GVs. This was exemplarily confirmed for OMVs from a non-pathogenic *E. coli* (strain 11/08) 1st GV, which transferred pESBL into 2nd GVs of different Enterobacteriaceae species (Figure 3). Taken together, these experiments demonstrated that the OMV-mediated dissemination of $bla_{CTX-M-15}$, bla_{TEM-1} and the associated pESBL is retained in Enterobacteriaceae vesiculants.

Influence of simulated intraintestinal conditions on OMV characteristics and OMV-mediated $bla_{CTX-M-15}$ transfer

Since the human large intestine is a major site of dissemination of ARGs,[47] we determined the influence of simulated human intraintestinal conditions on OMV-mediated $bla_{CTX-M-15}$ transfer. In parallel, we analysed effects of these conditions on related OMV characteristics including the OMV amount, size, DNA and protein concentrations and protein profiles. OMV production by strain C227-11ϕcu was significantly increased in both SIEM and SCEM, as demonstrated by 18.5-fold and 16.6-fold increases of OMV counts (particles/mL) determined by NTA and 4.4-fold and 4.8-fold increases of OMV protein concentration in OMVs-$_{SIEM}$ and OMVs-$_{SCEM}$, respectively, compared with OMVs-$_{LB}$ (Figure 4a and c). Sizes of OMVs-$_{SIEM}$ and OMVs-$_{SCEM}$ were similar to that of OMVs-$_{LB}$ both in NTA (Figure 4b) and in TEM (Figure 4e). Protein profiles of OMVs-$_{SIEM}$ and OMVs-$_{SCEM}$ showed no obvious differences from that of OMVs-$_{LB}$ (Figure 4d). Notably, OMVs-$_{SIEM}$ and OMVs-$_{SCEM}$ contained significantly higher quantities of DNA than OMVs-$_{LB}$, as demonstrated by significantly increased DNA/protein ratios in OMVs-$_{SIEM}$ and OMVs-$_{SCEM}$ compared with OMVs-$_{LB}$ (Figure 4c). PK/DNase-treated OMVs-$_{SIEM}$ and OMVs-$_{SCEM}$ were PCR positive for $bla_{CTX-M-15}$ and bla_{TEM-1}, indicating that the genes are located

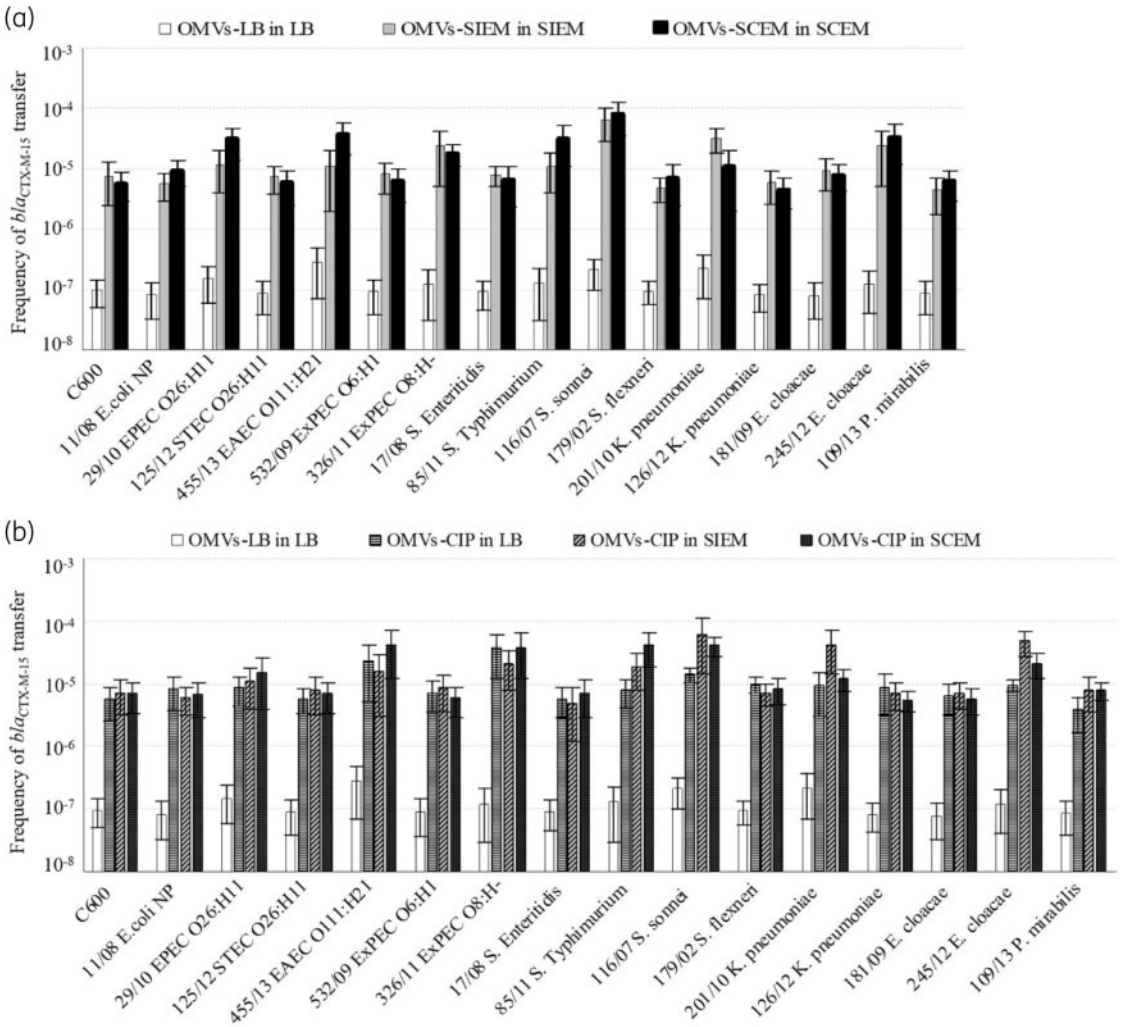

Figure 1. Frequencies of $bla_{CTX\text{-}M\text{-}15}$ transfer into Enterobacteriaceae via OMVs from *E. coli* O104:H4 strain C227-11ϕcu under different conditions. (a) Transfer via OMVs produced under standard laboratory conditions (LB broth; OMVs-$_{LB}$), in SIEM (OMVs-$_{SIEM}$) and in SCEM (OMVs-$_{SCEM}$) in the respective media. (b) Transfer via OMVs produced in LB broth (OMVs-$_{LB}$) and in LB broth with ¼ MIC of ciprofloxacin (OMVs-$_{CIP}$) in LB broth, SIEM or SCEM. Data are means ± SDs from three independent experiments. NP, non-pathogenic; EPEC, enteropathogenic *E. coli*; STEC, Shiga toxin-producing *E. coli*; EAEC, enteroaggregative *E. coli*; ExPEC, extraintestinal pathogenic *E. coli*.

intravesicularly (Table S11). These OMVs transferred $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1}$ into *E. coli* C600 and clinical Enterobacteriaceae under simulated intraintestinal conditions (Table S12). The frequencies of the $bla_{CTX\text{-}M\text{-}15}$ transfer via OMVs-$_{SIEM}$ and OMVs-$_{SCEM}$ ranged from $(4.5\pm2.7)\times10^{-6}$ to $(6.6\pm3.8)\times10^{-5}$ and from $(4.6\pm2.4)\times10^{-6}$ to $(8.3\pm4.7)\times10^{-5}$, respectively (Figure 1a and Table S12), being thus two orders of magnitude higher than those via OMVs-$_{LB}$ in LB broth (standard laboratory conditions) (Figure 1a). The numbers of OMVs-$_{SIEM}$ and OMVs-$_{SCEM}$ required to transfer 10 ng of DNA were 9.3×10^{9} and 8.3×10^{9}, respectively, which represents 0.6% of each OMV population (particles/mL); this is ∼14-fold lower than in OMVs-$_{LB}$ (Table S4). The vesiculants that acquired $bla_{CTX\text{-}M\text{-}15}$ via OMVs-$_{SIEM}$ and OMVs-$_{SCEM}$, respectively, expressed resistance to cefotaxime, ceftazidime and cefpodoxime and had an ESBL phenotype (Table S12). Altogether, these data indicated that the conditions encountered by Enterobacteriaceae in the human intestine substantially increase OMV production, OMV-associated DNA amount and the frequency of OMV-mediated $bla_{CTX\text{-}M\text{-}15}$ transfer.

Influence of ciprofloxacin on OMV characteristics and OMV-mediated $bla_{CTX\text{-}M\text{-}15}$ transfer

Ciprofloxacin, one of the antibiotics used during the *E. coli* O104:H4 outbreak,[48] significantly increased C227-11ϕcu OMV production, as demonstrated by both OMV counts (particles/mL) (Figure 4a) and protein concentration (Figure 4c). The mean diameter of OMVs-$_{CIP}$ did not significantly differ from those of the other OMV types (Figure 4b), but OMVs-$_{CIP}$ were more heterogeneous in size, as demonstrated by both NTA (Figure 4b) and TEM (Figure 4e). The protein profile of OMVs-$_{CIP}$ was similar but not identical to those of the other OMVs (Figure 4d). Like OMVs-$_{SIEM}$ and OMVs-$_{SCEM}$, OMVs-$_{CIP}$ contained significantly more DNA than OMVs-$_{LB}$ (Figure 4c). PK/DNase-treated OMVs-$_{CIP}$ were PCR positive for

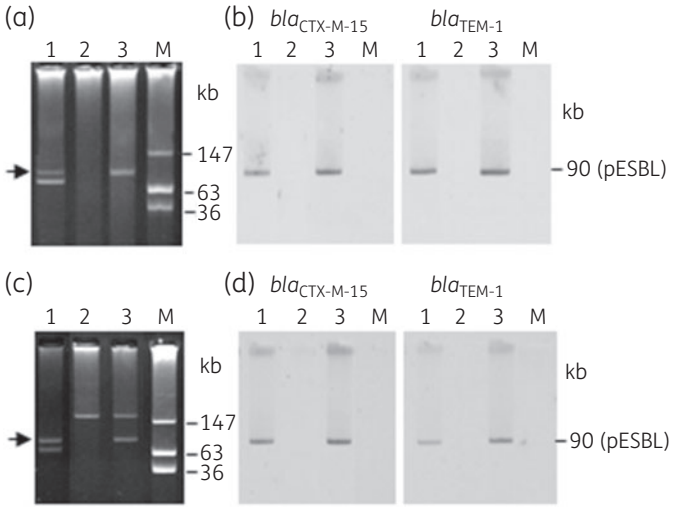

Figure 2. Transfer of pESBL via OMVs from *E. coli* O104:H4 strain C227-11ϕcu. Plasmid profiles (a and c) and plasmid hybridization with the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1}$ probes (b and d) of *E. coli* strain C227-11ϕcu (OMV donor), OMV recipients and the respective vesiculants that acquired pESBL via C227-11ϕcu OMVs. (a and b) Lane 1, C227-11ϕcu donor; lane 2, *E. coli* non-pathogenic 11/08 recipient; lane 3, *E. coli* non-pathogenic 11/08 vesiculant. (c and d) Lane 1, C227-11ϕcu donor; lane 2, extraintestinal pathogenic *E. coli* 532/09 recipient; lane 3, extraintestinal pathogenic *E. coli* 532/09 vesiculant. Lane M, molecular mass markers (plasmids from *E. coli* strain 39R861).[63] The arrows on the left sides of gels in panels (a) and (c) indicate pESBL. The sizes of plasmids that hybridized with the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1}$ probes (pESBL) are shown in panels (b) and (d).

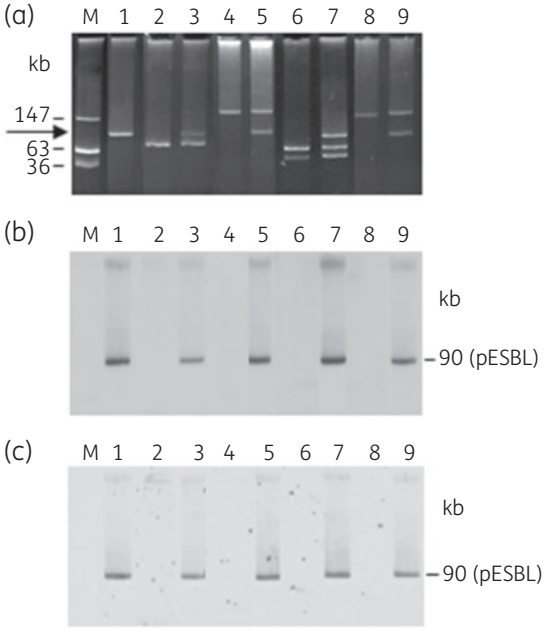

Figure 3. Transfer of pESBL via OMVs from a non-pathogenic *E. coli* 11/08 1st GV into 2nd GVs. Plasmid profiles (a) and plasmid hybridization with the $bla_{CTX\text{-}M\text{-}15}$ (b) and $bla_{TEM\text{-}1}$ (c) probe of *E. coli* 11/08 1st GV (OMV donor), OMV recipients and the respective 2nd GVs that acquired pESBL via *E. coli* 11/08 1st GV OMVs. Lane M, molecular mass markers (plasmids from *E. coli* strain 39R861).[63] Lane 1, *E. coli* 11/08 1st GV; lane 2, enteroaggregative *E. coli* 432/12 recipient; lane 3, enteroaggregative *E. coli* 432/12 2nd GV; lane 4, *K. pneumoniae* 378/10 recipient; lane 5, *K. pneumoniae* 378/10 2nd GV; lane 6, *Salmonella enterica* serovar Infantis 159/10 recipient; lane 7, *S.* Infantis 159/10 2nd GV; lane 8, *Shigella boydii* 451/07 recipient; lane 9, *S. boydii* 451/07 2nd GV. The arrow on the left side of the gel (a) indicates pESBL. The sizes of plasmids that hybridized with the $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1}$ probes (pESBL) are shown in panels (b) and (c).

$bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1}$ (Table S11), indicating that the genes were located inside OMVs, and transferred both genes into *E. coli* C600 and clinical Enterobacteriaceae under laboratory (LB broth) and simulated intraintestinal conditions (Figure 1b and Table S13). The frequencies of OMVs-$_{CIP}$-mediated $bla_{CTX\text{-}M\text{-}15}$ transfer were mostly two orders of magnitude higher than those mediated by OMVs-$_{LB}$ (Figure 1b) and in some cases they tended to be higher under simulated intestinal compared with laboratory conditions (Figure 1b and Table S13). The number of OMVs-$_{CIP}$ required to transfer 10 ng of DNA was 8.6×10^9, which represents 0.7% of the total OMVs-$_{CIP}$ population (particles/mL); this is similar to OMVs-$_{SIEM}$ and OMVs-$_{SCEM}$, but ∼12-fold lower than in OMVs-$_{LB}$ (Table S4). All vesiculants that acquired $bla_{CTX\text{-}M\text{-}15}$ via OMVs-$_{CIP}$ expressed resistance to cefotaxime, ceftazidime and cefpodoxime and had an ESBL phenotype (Table S13). Thus, ciprofloxacin significantly increased OMV production, OMV-DNA content and the frequency of OMV-mediated $bla_{CTX\text{-}M\text{-}15}$ transfer.

Discussion

The rapid global spread of CTX-M-producing Enterobacteriaceae strongly emphasizes the need for elucidation of the complex mechanisms involved in the horizontal transfer of $bla_{CTX\text{-}M}$ genes, which is a major factor implicated in the ongoing 'CTX-M pandemic'.[1] This is, to the best of our knowledge, the first report that OMVs, emerging vehicles of ARG transfer,[29–33] carry and disseminate the $bla_{CTX\text{-}M\text{-}15}$ gene encoding the most prevalent CTX-M ESBL among clinically important Enterobacteriaceae. This substantially extends the currently known mechanisms of the horizontal $bla_{CTX\text{-}M}$ transfer.[3,21,49] We also show that *E. coli* O104:H4 OMVs transfer into vesiculants the pESBL plasmid on which the $bla_{CTX\text{-}M\text{-}15}$ gene is, together with $bla_{TEM\text{-}1}$, located,[37–39] hereby demonstrating an alternative route of pESBL spread besides conjugation.[50] The latter observation is in agreement with OMV-mediated transfer of ARG-containing plasmids in other bacterial species.[27–30,32]

Another important and novel finding in our study is the significant increase of OMV-associated DNA content (Figure 4c) and the frequency of OMV-mediated $bla_{CTX\text{-}M\text{-}15}$ transfer (Figure 1a) under simulated intraintestinal conditions compared with laboratory conditions. Notably, the ∼100-fold increase in $bla_{CTX\text{-}M\text{-}15}$ transfer frequency under simulated intraintestinal conditions (Figure 1a) occurred even though the OMV amounts used for the transfer were ∼3-fold lower based on the protein concentration and ∼14-fold lower based on the proportions of OMV populations involved in the transfer than under laboratory conditions (Table S4). Together, these observations strongly support the involvement of OMVs in the dissemination of $bla_{CTX\text{-}M\text{-}15}$ and the associated pESBL *in vivo*, which is of particular importance since the large intestine is a major reservoir of ARGs and the site of their horizontal transfer.[47,51] The ability of OMVs to efficiently spread the $bla_{CTX\text{-}M\text{-}15}$-carrying

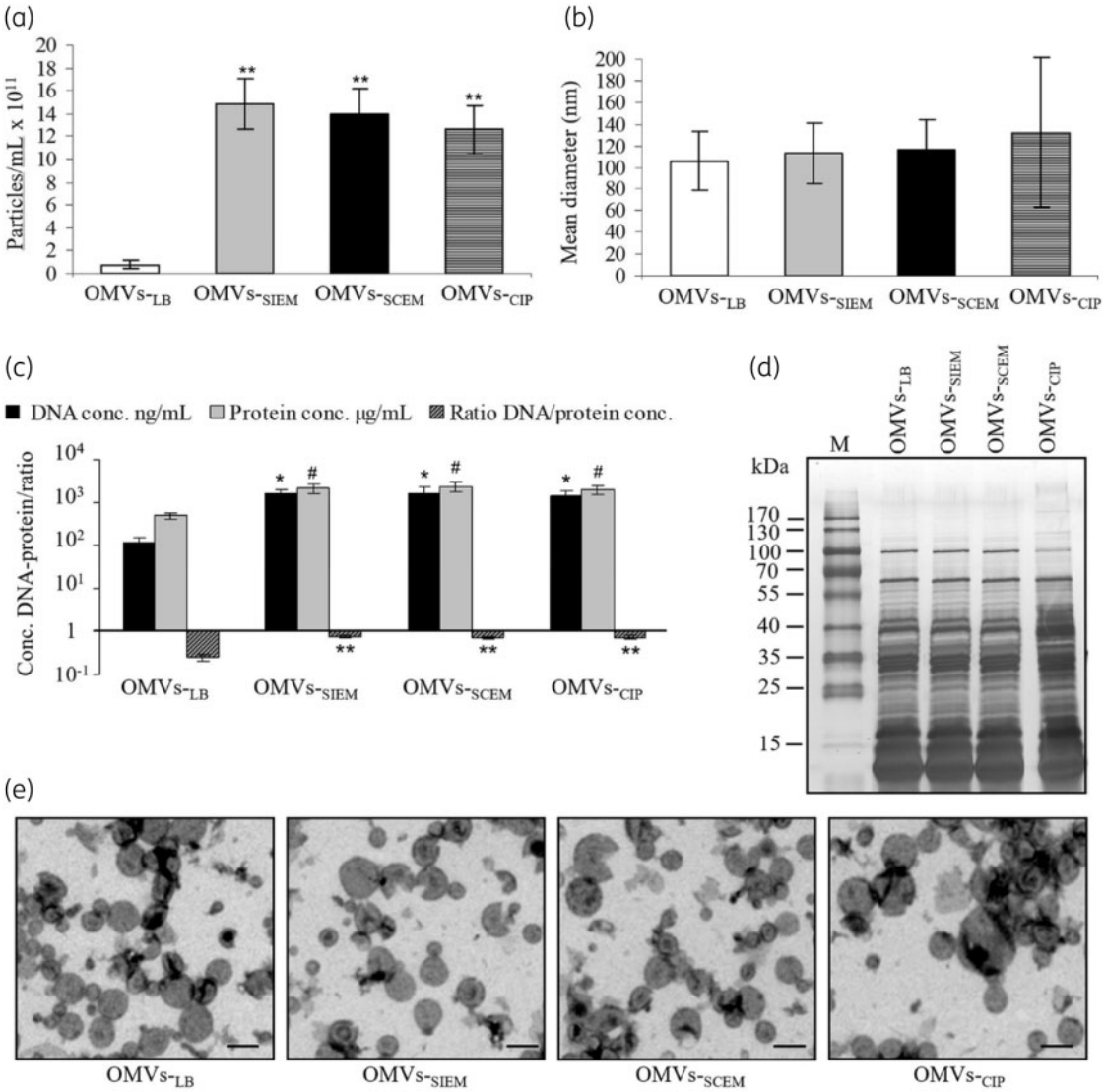

Figure 4. Comparative analysis of OMVs produced by strain C227-11ϕcu under different conditions. OMVs produced in LB broth (OMVs-$_{LB}$), SIEM (OMVs-$_{SIEM}$), SCEM (OMVs-$_{SCEM}$) and LB broth with 1/4 MIC of ciprofloxacin (OMVs-$_{CIP}$) were analysed for OMV counts (a) and OMV size (b) by NTA, for DNA and protein concentrations (c) by PicoGreen assay and Nanoquant reagent, respectively, for protein profiles (d) by gel electrophoresis followed by silver staining (ProteoSilver Plus Silver Stain Kit) and for OMV morphology (e) by TEM after negative staining. The values in (a), (b) and (c) are means ± SDs from three independent experiments. Images in (d) and (e) show one representative experiment; lane M in (d) contains a molecular mass marker (marker IV; Peqlab) with band sizes shown on the left side; bars in (e) are 100 nm. (a) **, significantly increased ($P < 0.001$) compared with OMV counts (particles/mL) of OMVs-$_{LB}$; (c) * and #, significantly increased ($P < 0.05$) compared with the DNA and protein concentration, respectively, of OMVs-$_{LB}$; **, significantly increased ($P \leq 0.001$) compared with the ng of DNA/µg of protein ratio of OMVs-$_{LB}$. Calculations were performed with Student's *t*-test.

plasmids in the human intestine is supported by: (i) OMV-mediated protection of intravesicularly located DNA from environmental DNases; (ii) OMV-mediated transfer of the $bla_{CTX\text{-}M\text{-}15}$-carrying pESBL into a broad spectrum of Enterobacteriaceae, likely due to the close similarity between the bacterial and OMV outer membrane, which allows OMV fusion with the bacterial surface and subsequent intrabacterial DNA delivery;[30,52,53] (iii) the ability of vesiculants that acquired the $bla_{CTX\text{-}M\text{-}15}$-carrying pESBL via *E. coli* O104:H4 OMVs to further spread the plasmid among Enterobacteriaceae, ensuring continuous $bla_{CTX\text{-}M\text{-}15}$ dissemination; and (iv) considerable stability of *E. coli* O104:H4 OMVs in simulated intestinal media and in human stool (M. Bielaszewska, unpublished data), which enables the OMV-associated DNA to persist in the environment, thus enhancing its chance to meet a suitable recipient. These observations, together with similar reports on ARG-carrying OMVs from other pathogens,[28–30,32,33] indicate that OMVs are, besides conjugative plasmids,[9,54,55] an additional powerful route for dissemination of ARGs in the human intestine.

Ciprofloxacin has, until recently, been an antibiotic of choice for the prophylaxis and treatment of traveller's diarrhoea.[56] However, increasing resistance[56] and increased colonization with ESBL-producing Enterobacteriaceae, often those harbouring $bla_{CTX\text{-}M\text{-}15}$,

after ciprofloxacin administration[57,58] have resulted in restricted recommendations for its use.[56] The latter observation is of particular interest in the context of our finding that OMVs produced in the presence of ciprofloxacin transferred $bla_{CTX-M-15}$ into Enterobacteriaceae up to 100 times more frequently than OMVs produced under laboratory conditions (Figure 1b), despite the fact that the amount of OMVs-$_{CIP}$ used was ~3-fold lower based on the protein concentration and ~12-fold lower based on the proportion of OMV population required for the transfer than that of OMVs-$_{LB}$ (Table S4). This effect is most likely due to the ciprofloxacin-mediated DNA damage and subsequent increase of OMV production as a result of activation of the SOS response.[45,59] Ciprofloxacin-induced activation of the SOS response has been implicated in transfer of an integrating conjugative element encoding MDR in *Vibrio cholerae*[60] and in phage-mediated transfer of kanamycin resistance in salmonellae.[61] Our finding of the ciprofloxacin-induced increase of $bla_{CTX-M-15}$ transfer via OMVs thus extends the number of pathways by which this drug promotes horizontal spread of ARGs. Notably, this can occur even with a subinhibitory ciprofloxacin concentration (1/4 MIC), which is ~2000-fold lower than that achieved in the intestine after routine oral doses.[62] Together, these data support the recommended restrictions on ciprofloxacin use.[56,58] Moreover, our data, together with the report that gentamicin increased the frequency of ampicillin resistance transfer via OMVs in *Acinetobacter baylyi*,[30] indicate that modulation of OMV-mediated ARG transfer via antibiotic stress encountered by bacteria during therapy is a novel way in which antimicrobials may substantially contribute to the dissemination of ARGs.

In conclusion, our data demonstrate that OMVs are efficient vehicles for dissemination of the $bla_{CTX-M-15}$ gene and the associated pESBL plasmid among Enterobacteriaceae and may represent an as-yet-unrecognized mechanism of $bla_{CTX-M-15}$ transfer in the human intestine.

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Transparency declarations

None to declare.

Supplementary data

Tables S1 to S13 and Figures S1 and S2 are available as Supplementary data at *JAC* Online.

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