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Extensive antimicrobial resistance mobilization via multicopy plasmid encapsidation mediated by temperate phages

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Objectives: To investigate the relevance of multicopy plasmids in antimicrobial resistance and assess their mobilization mediated by phage particles

Methods: Several databases with complete sequences of plasmids and annotated genes were analysed. The 16S methyltransferase gene *armA* conferring high-level aminoglycoside resistance was used as a marker in eight different plasmids, from different incompatibility groups, and with differing sizes and plasmid copy numbers. All plasmids were transformed into *Escherichia coli* bearing one of four different lysogenic phages. Upon induction, encapsidation of *armA* in phage particles was evaluated using qRT–PCR and Southern blotting.

Results: Multicopy plasmids carry a vast set of emerging clinically important antimicrobial resistance genes. However, 60% of these plasmids do not bear mobility (MOB) genes. When carried on these multicopy plasmids, mobilization of a marker gene *armA* into phage capsids was up to 10000 times more frequent than when it was encoded by a large plasmid with a low copy number.

Conclusions: Multicopy plasmids and phages, two major mobile genetic elements (MGE) in bacteria, represent a novel high-efficiency transmission route of antimicrobial resistance genes that deserves further investigation.

Introduction

Antimicrobial resistance is recognized today as one of the most serious threats to human health.[1] Understanding the mechanisms underlying the emergence and transmission of antimicrobial resistance genes in humans, animals and the environment has become a major goal in microbiological studies worldwide.[2–4] Research on horizontal gene transfer in bacteria is of special relevance, as it leads to the rapid spread of known and novel antimicrobial resistance genes. Although a lot of effort has been focused on plasmids and their spread through conjugation or transformation, little is known regarding the relationship between plasmids and phages, two major mobile genetic elements in bacteria. We have recently identified fragments of phages in small multicopy plasmids (MCPs) in enterobacteria.[5] This led us to hypothesize that plasmids in general, but probably with more frequency in MCPs, could be transferred between bacteria by phage transduction. Several other facts support this notion: (i) many MCPs lack mobility (MOB) genes, but are widely spread in bacterial populations;[5] (ii) each plasmid is present in the cytoplasm in multiple copies, which can rise to 100 copies/cell during antibiotic treatment,[6] increasing the theoretical probability of a plasmid or plasmid-borne gene being encapsidated during general transduction; (iii) antimicrobial resistance (AMR) genes normally found on plasmids have been identified in phage particles;[7,8] and (iv) like other bacterial DNA, plasmids can be transferred through generalized transduction.[9–12]

Great attention was focused on MCPs in the 1970s, as they were, and are, essential in biotechnology and gene manipulation.[13] However, understanding of the relevance of MCPs in AMR is more recent. We showed that MCPs are responsible for β-lactam resistance in several pathogens, and that MCPs bearing different resistance genes can stably coexist in a single bacterium, giving

rise to a novel bacterial strategy for multidrug resistance.[14] Further, it is widely known that duplication of genes facilitates gene innovation.[15] Notably, a single SNP in the replication origin of an MCP can give rise to a novel variant, with a different plasmid copy number and fitness cost,[16] showing how easily gene innovation and spread are facilitated by MCPs. In addition, AMR genes can undergo mutations and further selection in MCPs.[17]

To test our hypothesis, we have first analysed the relevance of MCPs in bacteria. Second, we have assessed AMR genes present in MCPs. Third, we have tested the efficiency of phage encapsidation of an AMR gene borne on different genetic platforms. To do this, we have used the same AMR gene, *armA*,[18–20] as a marker in eight different plasmids of different sizes (≈5 kb to ≈80 kb), different plasmid copy numbers (1–45), and belonging to different incompatibility groups. To discard generalized transduction events, we have used four temperate phages that lysogenize the donor strains. The amount of *armA* in phage particles has been quantified after phage induction and the results of packaging of the different plasmids have been compared.

Materials and methods

In silico analysis of plasmids in bacteria

To examine the distribution of plasmids based on their predicted mobility, 16702 plasmid sequences were retrieved from the NCBI nucleotide database, using the PLSDB web server for screening purposes.[21] Only plasmid records with circular sequences or complete assemblies were selected. Computational prediction analysis of the mobilization capacity was performed based on the presence of relaxases and mating pair formation systems using the MOB-typer tool included in the MOB-suite software.[22] Plasmids lacking a relaxase were classified as 'non-MOB', while the presence of a type IV secretion system (T4SS), involved in mating pair formation during conjugation, enabled us to separate potentially 'conjugative' and 'MOB' plasmids.

Analysis of AMR genes in small plasmids

Small plasmids (ColE1, ColE10 and IncQ) were identified based on plasmid replicon sequences obtained from the PlasmidFinder database,[23] using the BLASTn algorithm to look for DNA homologies in the GenBank database. Complete plasmid accessions were retrieved from the NCBI nucleotide database, using an Entrez query with filters to exclude incomplete or non-plasmid sequences. A local, command-line version of AMRfinder tool (https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder/) and ResFinder tool[24] with default parameters was used to identify AMR genes in MCPs, allowing enumeration and classification of the genes according to the antimicrobial class they conferred resistance to, along with the plasmid size and GenBank accession number.

Bacteria, strains and culture conditions

Eight different plasmids all carrying the same resistance marker, *armA*, were used in this study: five conjugative plasmids larger than 47 kb that naturally carry *armA*, belonging to different incompatibility groups (IncM2, IncFII, IncR, IncX1 and IncN), and three vectors smaller than 12 kb (pACYC184, pCR2.1 and pUC18) into which *armA* was cloned (Table 1).

The large plasmids were isolated from WT specimens from different origins that contained the *armA* gene and were pan-resistant to aminoglycosides. The plasmids were sequenced using Illumina and Nanopore technology (see below) and the complete nucleotide sequence was deposited in the ENA database together with the corresponding metadata on their origin and date of isolation (Table 1). Construction of pUC18::*armA* is as described by Gonzalez-Zorn *et al.*[18] Cloning using the poly(A) tail was used for pCR2.1::*armA* only. To clone *armA* into pACYC184, the gene was amplified with armA400 and armAR, then the product was digested with EcoRI and cloned into the EcoRI site of pACYC184 (Table S1, available as Supplementary data at *JAC* Online).

Table 1. Plasmids used in this study

Plasmid	Group	Size (bp)	Copy number	Reference
pACYC184::*armA*	p15A	5682	8.34±1.07	This work
pCR2.1::*armA*	ColE1-like	4929	23.11±1.7	This work
pUC18::*armA*	ColE1-like	11680	41.78±4.23	18
pB1362	IncM2	77297	2.5±0.13	ERZ1101256
pB2920	IncFII	77960	0.78±0.22	ERZ1079030
pB2948	IncR	47627	1.11±0.24	ERZ1079031
pB2954	IncX1	77121	1.32±0.14	ERZ1079029
pMUR050	IncN	56634	0.99±0.03	AY522431.4

Escherichia coli strain WG5 lysogenic for Stx phages 933W, 312 or 557,[25] or a Cdt phage,[26] and *E. coli* strain DH5α lysogenic for Stx phages 933W, 312 or 557,[25] were used as recipient strains for the plasmids mentioned above to evaluate the packaging of plasmids or plasmid fragments in phage particles. Strain WG5 is a nalidixic acid-resistant mutant of *E. coli* C (also known as strain CN) and does not contain resistance genes or complete inducible prophages.[27] DH5α is a *recA*-negative *E. coli* K-12 derivative.[28] Stx phages 933W, 312 and 557 are three different phages of the *Podoviridae* morphological type carrying the Shiga toxin (*stx*) gene, while Cdt phage is from the *Myoviridae* morphological type carrying the cytolethal distending toxin (*cdt*) gene.[26] Stx phages and Cdt phages are temperate *cos* phages that package a fragment of DNA delimited between two *cos* sites and therefore are not prone to packaging DNA through generalized transduction.[29] All lysogens were transformed with MCPs (pACYC184, pCR2.1 or pUC18) and low-copy plasmids (IncM2, IncFII or IncR). Additionally, low-copy plasmids IncX1 and IncN were used to transform some of the lysogens and were also added to the study. Bacterial strains and phages were selected to study whether differences related to the phages or to the host strain could be factors influencing the encapsidation of the different plasmids.

Complete sequence of plasmids with Illumina and MinION technology

Genomic DNA extraction and purification were performed using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA). DNA quality and concentration were measured by NanoDrop (Thermo Fisher Inc., Waltham, MA, USA) and Qubit (Invitrogen Corp., Carlsbad, CA, USA) devices. Short paired-end reads were generated on a HiSeq 2500 platform (Illumina Inc., San Diego, CA, USA). Sequences were processed for subsequent analysis by checking their quality with FastQC version 0.11.3 and trimming end nucleotides of low quality using Trimmomatic version 0.33. Genomes were sequenced using a MinION device (Oxford Nanopore Technologies Ltd., Oxford, UK). Genomic libraries were generated following the 1D Native barcoding genomic DNA protocol, with EXP-NBD103 and SQK-LSK108 kits (Oxford Nanopore Technologies Ltd.), and sequencings were run in a FLO-MIN106 flow cell. Sequencing reads were base-called with MinKNOW software (Oxford Nanopore Technologies Ltd.), demultiplexing was carried out with the Fastq Barcoding workflow of the Epi2Me interface (Metrichor Ltd., Oxford, UK) and trimming of adaptors and barcodes from the reads was assessed by Porechop version 0.2.3. Hybrid assemblies with short and long sequencing reads were achieved using Unicycler version 0.4.0. Closed genomic structures were assessed by Bandage version 0.8.1 and annotated by

Prokka version 1.5. Antibiotic resistance genes and plasmid incompatibility groups present in each of the genomic structures were identified using Bandage and applying the ResFinder and PlasmidFinder databases, respectively.

Plasmid copy number quantification and qPCR for armA

The average number of copies of each plasmid was determined by quantitative PCR (qPCR) as described by San Millan *et al.*[6] in triplicate from three independent DNA extractions from DH5α-557. DNA extractions were carried out from 2 mL of LB broth cultures, at an OD_{600} of approximately 0.6, using a QIAamp DNA Mini Kit (Qiagen, USA). The amount of DNA was quantified using Qubit (Invitrogen Corp., Carlsbad, CA, USA). Since restriction enzyme-digested total DNA is a better template source than non-digested total DNA for plasmid quantification by qPCR,[30] 100 ng of DNA from each sample was digested using FastDigest PstI (Thermo Fisher Scientific, USA) for 1 h at 37°C. The desired amplification products do not contain the PstI target sequence. We developed a specific qPCR for the *armA* gene carried in all the plasmids. In order to determine the average plasmid copy number per chromosome, the monocopy gene *uidA* was amplified to compare the ratio of plasmid to chromosomal DNA (Table 1). A standard curve of each reaction was generated by performing qPCR with five 5-fold dilutions of template DNAs in triplicate working range of DNA concentration, ≈0.2 ng/μL to 320 fg/μL, from which the efficiency of the reaction was calculated. qPCR was performed using a My iQ single-colour real-time PCR detection system (Bio-Rad, USA) and the Bio-Rad iQ SYBR Green Supermix (Bio-Rad, USA) at a final concentration of 20 pg/μL. The amplification conditions were as follows: initial denaturation for 10 min at 94°C, followed by 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 49.3°C (*armA*) or 55°C (*uidA*), and extension for 1 min at 72°C. To calculate plasmid copy number per chromosome, we used the formula described previously:[31] $cn = (1+E_c)^{Ctc}/(1+E_p)^{Ctp} \times S_c/S_p$, where cn is the plasmid copy number per chromosome, S_c and S_p are the sizes of the chromosomal and plasmid amplicons in bp, respectively, E_c and E_p are the efficiencies (relative to 1) of the chromosomal and plasmid qPCRs, respectively, and Ctc and Ctp are the threshold cycles of the chromosomal and plasmid reactions, respectively. The *armA* qPCR assay[30] was designed using the sequence of *armA* in plasmid pMUR050 (AY522431.4) from an *E. coli* pig isolate.[18] pMUR050 was also used to generate standard curves.[8] Only qPCR reactions with an efficiency between 95% and 100% were considered as significant. The *armA* qPCR assay has an average efficiency of 98.4% and a detection limit of 2.74 gene copies. qPCR amplification was conducted with 1 μL of extracted packaged DNA under standard conditions in a Step One Real-Time PCR System (Applied Biosystems, Life Technologies).[30] Student's *t*-test was used to assess statistical significance.

Phage induction and isolation of phage particles

Cultures (10 mL) of each lysogen were grown on LB broth to the mid-exponential phase (OD_{600} of 0.3) for optimal phage induction and treated with mitomycin C (final concentration 0.5 μg/mL), which induces the SOS cell response and drives the induction of the lytic cycle of the phage and the production of phage particles. Cultures were incubated overnight in the presence of mitomycin C at 37°C with shaking in the dark because mitomycin C is light sensitive. After incubation, phage particles were extracted as previously described.[7] Briefly, the cultures were filtered through low protein-binding 0.22 μm pore-size membrane filters (Millex-GP, Millipore, Bedford, MA, USA) to eliminate cell debris, treated with chloroform (1:10 v/v) to break cell membranes and possible membrane vesicles that might contain nucleic acids, and treated with DNAse I (100 units/mL; Sigma–Aldrich, Spain) for 1 h at 37°C to eliminate non-packaged DNA. At this stage, the phage suspension was used for transmission electron microscopy observations. To confirm the removal of non-packaged DNA, an aliquot of the sample was taken after DNase I treatment and before its de-encapsidation with proteinase K and used to confirm the absence of *armA* by qPCR.[7] Different controls were performed to confirm the stability of the DNase I and its inactivation,[32] before extracting packaged DNA by proteinase K digestion, and by phenol chloroform purification as previously described.[32,33] Extracted packaged DNA was used for qPCR amplification and Southern blot analysis as described below. The Pearson correlation coefficient (*r*) was calculated by two-tailed correlation test in order to determine the linear association between the plasmid copy number and phage encapsidation using R version 3.6.1 and $P \leq 0.05$ as the critical level of significance.

Southern blotting

The DNA fragment of the *armA* and the different probes resulting from amplification with the respective primers (Table S1) were labelled with digoxigenin. The probe was labelled by incorporating digoxigenin-11-deoxy-uridine-triphosphate (Roche Diagnostics. Barcelona, Spain) during PCR as described.[34]

For Southern blot hybridization a higher DNA concentration was required, and for this purpose we extracted packaged DNA from 100 mL cultures of strains WG5-933W, WG5-312 and DH5α-557, selected for being representative of three different phages in two different strains, and containing the different plasmids. We obtained a final volume of 50 μL of DNA that was digested with S1 nuclease (Promega Co., Inc., MA, USA) and analysed by separation on 0.7% agarose gels in 0.5-fold concentrated Tris borate EDTA buffer and stained with ethidium bromide.

Digested DNA was transferred to nylon N+ membranes (Hybond N+, Amersham Pharmacia Biotech, Spain) by capillary blotting.[35] The presence of *armA* and the plasmids was determined in all the digested phage DNA by Southern blot hybridization with the digoxigenin-labelled probes prepared as described above. Stringent hybridization was achieved with the DIG DNA Detection Kit (Roche Diagnostics, Barcelona, Spain) according to the manufacturer's instructions.

After obtaining *armA* results, the membranes were incubated with dimethylformamide, heated at 60°C, washed with 0.2 N NaOH and 0.1% SDS solution at 37°C for 10 min to strip the *armA* probe and hybridized again,[35] using the respective digoxigenin-labelled probes targeting regions not close to *armA* in each plasmid (Table S1). The probes targeting other regions in the small plasmids are located opposite to the insertion site of *armA*. In large plasmids, the probe is located 5 kbp upstream of *armA*.

Results and discussion

Plasmid sizes and mobilization genes

Plasmids are the major vehicle of AMR genes. To evaluate the sizes and potential mobilization capacity of plasmids, we analysed all fully sequenced plasmids available, corresponding to ≈16700 complete circularized plasmid sequences (see Materials and methods). The plot of number of plasmids versus plasmid size is shown in Figure 1. Plasmids follow a bimodal distribution, with a clear differentiation of small plasmids (<20 kb) and large plasmids (>20 kb). It is generally accepted that the main dissemination strategy of plasmids is conjugation. Conjugative plasmids possess the full genetic machinery encoding a T4SS and the relaxases to undergo self-transmission, which is known to require ≈25 kb of genetic information.[36,37] We therefore analysed how many plasmids possess a complete conjugative apparatus. In line with their small size, none of the small plasmids are predicted to be conjugative. Interestingly, only 44.2% of the large plasmids possess a full T4SS.

Another set of plasmids carry just a relaxase that enables conjugation using the T4SS of a co-resident conjugative plasmid.[38,39] We call them here MOB plasmids and they constitute 40.5% of

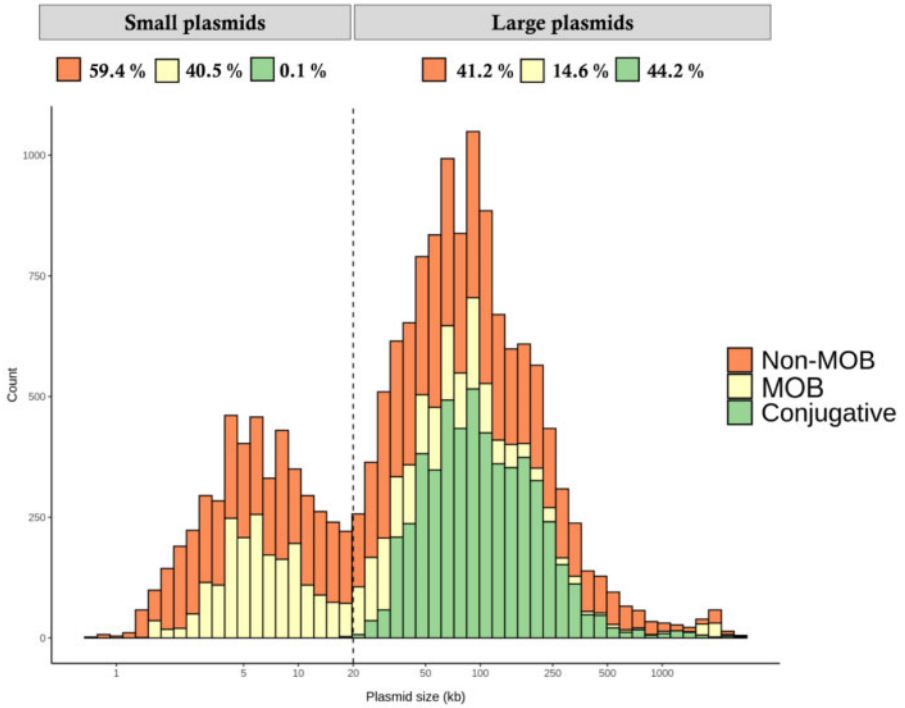

Figure 1. Histogram of 16 702 fully sequenced and circular plasmids. Plasmids follow a bimodal distribution: small plasmids (<20 kb) and large plasmids (>20 kb). Plasmids were predicted to be conjugative (with T4SS), MOB (without T4SS, with a MOB gene) and non-MOB (without T4SS or a MOB gene). Note that most plasmids are not transmissible. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

small plasmids. Among the large plasmids, 14.6% have MOB genes and no T4SS. Consequently, the majority of plasmids are non-MOB plasmids (41.2% of all large plasmids and 59.4% of small plasmids). However, small plasmids do not need to have MOB genes to be efficiently mobilized through conjugation, as it has recently been found that non-MOB plasmids with an origin of transfer (*oriT*) can be conjugated by relaxases encoded in co-resident plasmids acting in *trans*.[37,40]

Small plasmids confer resistance to most antimicrobial classes

The genes borne on small plasmids were listed and classified according to the antimicrobial class they conferred resistance to (Table 2). The table shows different resistance genes found in small plasmids belonging to families of MCPs according to the current literature. These plasmids encode resistance to aminoglycosides, β-lactams, macrolides, lincosamides and streptogramins (MLSs), phenicols, sulphonamides, tetracycline and trimethoprim. The mean plasmid size of all MCPs listed here was 8.3±3.6 kb (SD). Interestingly, emerging genes conferring resistance to clinically relevant or last-resort antibiotics such as fluoroquinolones (*qnrS2* and *qnrB19*), carbapenems, (bla_{KPC-2}, bla_{KPC-3}, bla_{BKC-1} and $bla_{OXA-656}$) and colistin (*mcr-4* and *mcr-5*) were also found on small plasmids, highlighting the relevance of these small mobile genetic elements in AMR. Although most of these MCPs (such as pCCK647[41] or pMCR_R3445,[42]) carry a single resistance gene, other resistance genes were aligned in tandem (like in pB1005,[43]), were found in transposon elements,[14] or were contained in a complete integron (like in pQ7,[44]). Thus, potential transfer of these replicons would lead to resistance not only to a single antimicrobial class, but to several different ones, giving rise to MDR in the recipient bacterium. The bacterial hosts with small plasmids are classical human pathogens (*Haemophilus influenzae*, Gram-negative opportunistic or pathogenic bacteria, *E. coli*, *Klebsiella pneumoniae* or *Pseudomonas* spp.) and strict animal pathogens such as *Haemophilus (Glaesserella) parasuis*.

High-efficiency packaging of DNA from MCPs in phages

Due to their relevance in AMR, high frequency and lack of mobilization genes, the interaction of MCPs with phages was further investigated. The different *E. coli* genetic backgrounds (*E. coli* WG5 or DH5α) were lysogenic for different phages (*Podoviridae* Stx phages 933W or 312 or *Myoviridae* Cdt phage). All lysogens were transformed with the same set of plasmids, all bearing the 16S rRNA methyltransferase gene *armA*, conferring resistance to all clinically relevant aminoglycosides (Table 1). The large plasmids belonged to the IncFII, IncM2, IncR, IncN and IncX1 incompatibility groups, whereas the MCPs belonged to the ColE1 and p15A families (Table 1). The copy number of all plasmids was determined using qPCR and a chromosomal monocopy gene as a marker (see Materials and methods). The plasmid copy number showed that plasmid pUC18 had the highest copy number, with 42 copies/cell, followed by pCR2.1 (23 copies/cell) and pACYC184 (8 copies/cell). All large plasmids, however, had a copy number that ranged between 1 and 3 (Table 1).

Table 2. Antimicrobial resistance genes located on small plasmids

Resistance gene	Plasmid	Plasmid size (bp)	GenBank accession no.
Aminoglycoside			
aac(6')-Ib3	pCHE-A1	8201	KX244760.1
aac(6')-Ib4	pJF-789	9016	KX912254.1
aac(6')-Ib-cr	pMdT1	5931	JX457478.1
ant(2'')-Ia	pVAS24-VEB	9159	KX575838.1
aph(3')-Ia	pMR0716_ColRNAI	5310	CP018108.1
aph(3')-IIIa	pCCK411	5265	FR798946.1
aph(3'')-Ib	pB1005	4237	FJ197818.1
aph(3')-Via	pKPN535a	14873	MH595533.1
aph(6)-Id	pLC1477_18-3	8398	CP035011.1
aadA1	pCCK343	5415	FR687372.1
aadA14	pCCK647	5198	AJ884726.1
β-Lactam			
bla_{TEM-1a}	pEC404/03-4	8599	AP014807.1
bla_{TEM-1b}	p0.1229_3	12894	CP028323.1
bla_{TEM-2}	pEC886	9261	HQ659758.1
$bla_{TEM-116}$	MK753226	5607	MK753226.1
$bla_{TEM-144}$	pST12	8275	HG428760.1
bla_{BKC-1}	p60136	9786	KP689347.1
$bla_{CTX-M-3}$	pPSTRAS1	9910	MH463250.1
$bla_{CTX-M-14}$	RCS63_p	22308	LT985269.1
$bla_{CTX-M-17}$	pIP843	7086	AY033516.1
bla_{GES-1}	pQ7	9042	FJ696404.1
bla_{GES-5}	pCHE-A	7560	EU266532.1
bla_{GES-7}	pPCMI3	9448	MH569711.1
bla_{SHV-12}	RCS35_pII	14365	LT985233.1
bla_{KPC-2}	pKPN535a	14873	MH5955331
bla_{KPC-3}	pKPC_Kp02	11930	KX348145.1
bla_{KPC-21}	pUR19829-KPC21	12748	MH133192.1
bla_{CMY-2}	pEC5106	14845	KY612499.1
bla_{CMY-4}	pQEL231	6925	KP205272.1
$bla_{OXA-256}$	pUL3AT	9005	HE616889.1
$bla_{OXA-655}$	pQGU16	14146	MH718732.1
$bla_{OXA-656}$	pQGU13	15473	MH718731.1
bla_{ROB-1}	pB1000	4613	GU080062.1
bla_{ROB-2}	pKKM48	4323	MH316128.1
bla_{VEB-18}	pVAS24-VEB	9159	KX575838.1
Polymyxin			
mcr-4.1	pMCR_R3445	8749	MF543359.1
mcr-4.2	pMCR-4.2_AB243	9513	MG800340.1
mcr-4.3	pEn_MCR4	8639	MH061380.1
mcr-5.1	pSE11-03671	8936	MK360094.1
mcr-5.2	pEC2380	11708	MG587004.1
Fluoroquinolone			
qnrS2	pPH18	6388	KU644672.1
qnrB19	pPAB19-2	3082	JN979787.1
Macrolide, lincosamide and streptogramin B			
lnu(G)	pIE1115	10687	AJ293027.1
erm(42)	A1_180	7406	CP040385.1
Phenicol			
catA1	pDT4	3716	HF570110.1
catA3	pMHSCS1	4992	AJ249249.1
catB2	pJR1	6792	AY232670.1
floR	p518	3937	KT355773.1
Streptothricin			
sat2	pCCK343	5415	FR687372.1
Sulphonamide			
sul2	pB1005	4237	FJ197818.1
Tetracycline			
tet(A)	pLC1477_18-3	8398	CP035011.1
tet(B)	pHS-Tet	5147	AY862435.1
tet(C)	pIS2	6349	AY167049.1
tet(G)	pJR1	6792	AY232670.1
tet(H)	pB1018	6074	JQ319774.1
tet(L)	pCCK3259	5317	AJ966516.1
tet(O)	pB1006	6033	FJ234438.1
Trimethoprim			
dfrA1	pCCK343	5415	FR687372.1
dfrA14	pABC-3	6779	KT988306.1
dfrB3	pPCMI3	9448	MH569711.1

bla_{OXA}-256/655/656 belong to the OXA-10 class D family.

Upon induction with mitomycin C and isolation of capsids, all purified phage lysates were shown to have the *armA* gene (Figure 2). Non-lysogenic DH5α strains transformed with the same set of plasmids did not show the detection of *armA* in an extract prepared in the same way as for phage (data not shown). Interestingly, the encapsidation rate of *armA* was significantly higher when the genetic platform of *armA* was an MCP than when it was carried on a large low-copy plasmid ($P < 0.0001$). Encapsidation, when *armA* was borne on MCPs such as pUC18, was up to 4 logarithmic units more efficient than when *armA* was borne on a large plasmid like IncR (Figure 2).

Southern blot analyses were performed with the DNA isolated from the phage fraction purified from some of the lysogens. We selected those showing enough encapsidated DNA to perform Southern blotting. As probes, an internal fragment of *armA* and probes targeting another region in each plasmid were used (Table S1). After S1 nuclease digestion to remove chromosomal DNA, a positive signal was obtained with both probes in fragments corresponding to 1.6 to >3 kb, confirming that in the phage fraction, not only *armA* but also other fragments of plasmid DNA were encapsidated (Figure S1). No complete plasmids, only shorter fragments, were detected in the Southern blots (Figure S1). Fragmentation of the DNA could be caused during the extraction process or due to S1 nuclease, which might more frequently cut packaged coiled plasmidic DNA than non-packaged plasmids. It could also be a mechanism for encapsidation, which must be required to package some of the large plasmids of >70 kb, since the phage capsid allows 62 kb for the three Stx phages or 34 kb for the Cdt phage.

Our selection of S1 nuclease may not be optimal, but it allowed the exclusion of chromosomal DNA, confirming the plasmidic nature of the packaged DNA. Moreover, digestion

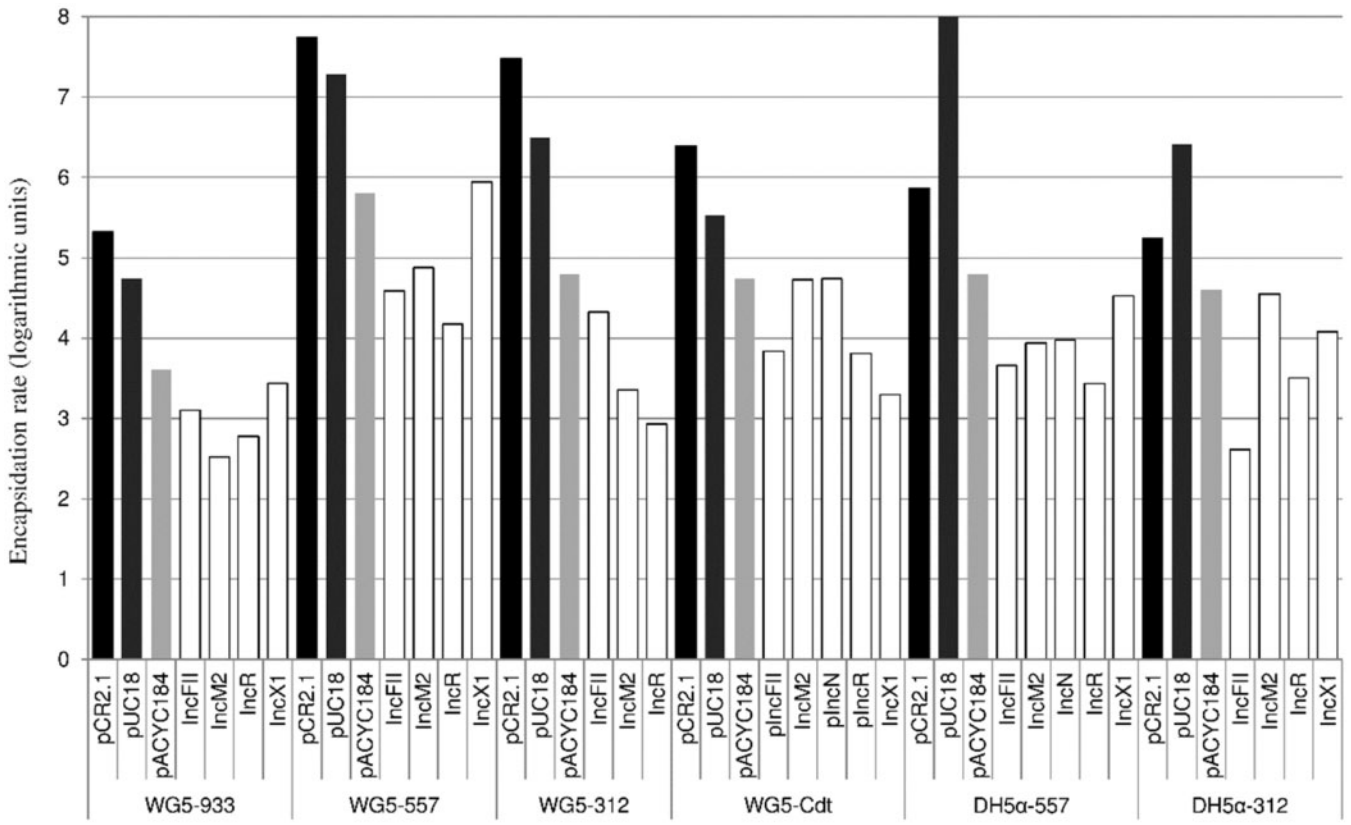

Figure 2. Representative experiment showing the detection of *armA* in the phage DNA fraction of *E. coli* strains WG5 and DH5α lysogenized with Stx phages 933W, 557 or 312 or Cdt phage and DH5α lysogenized with Stx phages 933W or 312 transformed with the different plasmids. Shaded bars correspond to MCPs and white bars to large, low-copy-number plasmids.

of packaged DNA using other endonucleases resulted in long DNA smears (data not shown).

Phages are able to encapsidate and transfer AMR genes.[45–47] This takes place not only in the environment, but also in the human gut, where antimicrobial treatment induces encapsidation of AMR genes.[48] Antibiotic resistance genes can be located chromosomally or in plasmids.[47] Generalized transduction of low- and high-copy plasmids has previously been shown with *Salmonella* phage P22, *Bacillus* phage SPP2 and *E. coli* phage Mu.[9–12] In addition, some strains harbour certain P1 phage derivatives that remain as plasmids during their life cycle without being integrated as prophages.[49] Our results confirm that AMR genes, when borne on plasmids, can use phages for potential mobilization. This is especially relevant in the case of MCPs, because they cannot transfer horizontally using conjugation. The difference from previous reports is that phages in our study use a *cos*-packaging mechanism, which eliminates the possibility of generalized transduction. The recently described chromosomal lateral transduction shows how phage packaging can mediate efficient transduction of complete genomes.[50] By lateral transduction, prophages packaging DNA through the *cos* mechanism can mobilize large fragments of chromosome, but no mobilization of plasmids has been reported by lateral transduction so far.

Southern blotting showed that fragmented plasmids were encapsidated. This could be attributable to fragmentation of DNA during the extraction process or due to S1 nuclease digestion, which may more frequently cut packaged plasmid DNA than non-packaged, coiled plasmids. Nevertheless, all MCPs (or their DNA fragments) were encapsidated with higher frequency than the low-copy plasmids. It should be considered that MCPs do not possess recognizable *pac* or *cos* sequences, classically used for transduction,[51] and therefore the plasmid packaging mechanism used by the *cos* phages in this study is not yet known.

The plasmid DNA would therefore mainly use phage capsids to disseminate, as most of them do not possess MOB genes. The potential encapsidation of the plasmids would appear to be independent of the phage induction rates mediated by mitomycin C. This is supported by the fact that, when using a DH5α host that is *recA* negative,[52] encapsidation rates were similar to those obtained with the *recA*-positive strain WG5. Also, the use of the Cdt phage, which has a higher level of spontaneous induction than the Stx phages,[26] did not show marked differences in terms of plasmid packaging. Therefore, the most significant difference between the plasmids is the size and copy number. In fact, there is correlation between the encapsidation rate and the copy number of the MCPs ($P = 0.0015$, Figure S2); *armA* in plasmid pACYC184, with the lowest copy number of 8 copies/cell, is potentially encapsidated with lower frequency than when borne on pCR2.1 or pUC18, with 23 and 44 copies/cell. Although at a lower rate, large plasmids could be transferred between bacteria through phages; up to 41% of all large plasmids analysed here also lack mobilization genes (Figure 1).

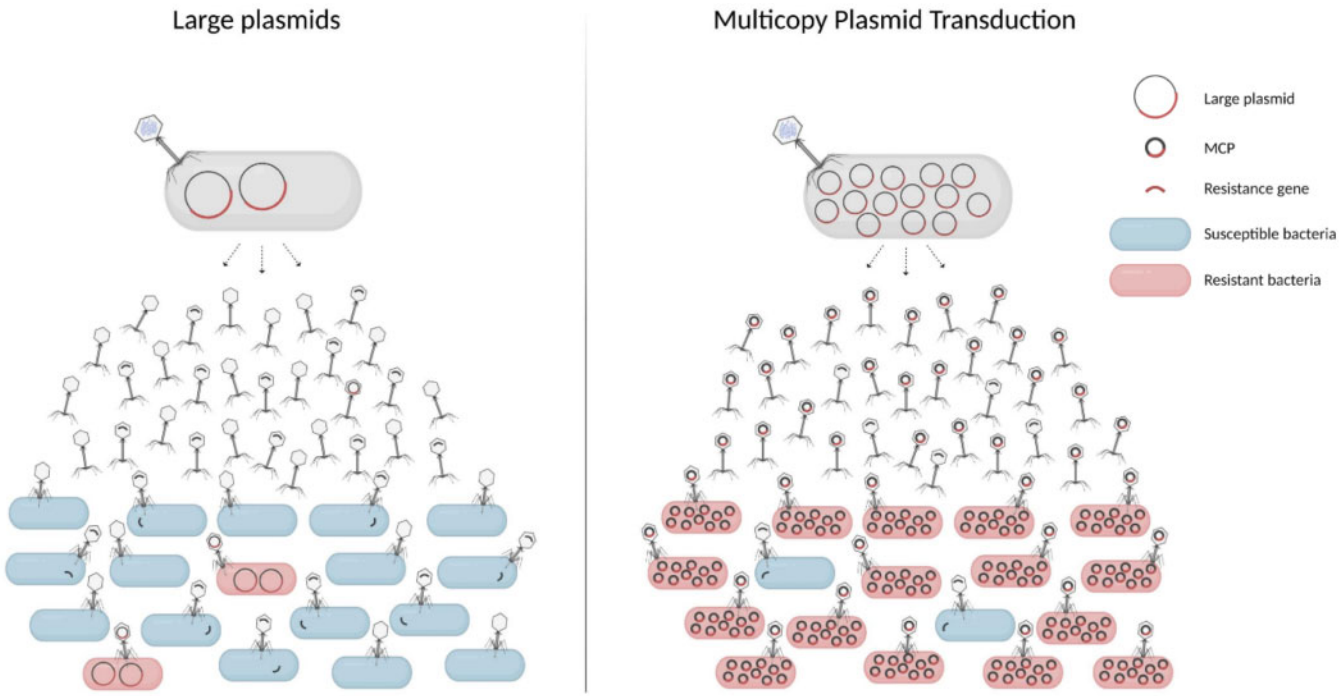

Figure 3. High efficiency spread of AMR through multicopy plasmid transduction. AMR genes borne on small MCPs (right) are encapsidated up to 10000 times more efficiently than when borne on large low-copy plasmids (left). As a consequence, the phages can disseminate over distance to transduce susceptible bacteria and transfer resistance genes. We propose a model where multicopy plasmid transduction is a major powerful route for AMR gene dissemination in nature, in which AMR spreads with high efficiency and over distance between bacteria from humans, animals and the environment. As the cargo of plasmids can also be other genes apart from AMR genes, this phenomenon represents a striking coordination between MGEs driving the evolution of bacterial populations. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

The use of phage therapy may need to take into account these results, to learn about the different mechanisms of AMR transfer, but also to focus efforts on the development of phage-based strategies that do not contribute to the spread of AMR genes.[53] Thus, our findings here suggest that MCP transduction (Figure 3) could be an extremely efficient means of mobilization of AMR genes, and although further investigations are needed to determine the mechanism of this phenomenon, our findings have key implications for plasmid evolution and the emergence and spread of AMR.

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Transparency declarations

None to declare.

Supplementary data

Table S1, Figures S1 and S2 are available as Supplementary data at *JAC* Online.

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