AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

Plasmid Characteristics Modulate the Propensity of Gene Exchange in Bacterial Vesicles

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ABSTRACT Horizontal gene transfer is responsible for the exchange of many types of genetic elements, including plasmids. Properties of the exchanged genetic element are known to influence the efficiency of transfer via the mechanisms of conjugation, transduction, and transformation. Recently, an alternative general pathway of horizontal gene transfer has been identified, namely, gene exchange by extracellular vesicles. Although extracellular vesicles have been shown to facilitate the exchange of several types of plasmids, the influence of plasmid characteristics on genetic exchange within vesicles is unclear. Here, a set of different plasmids was constructed to systematically test the impact of plasmid properties, specifically, plasmid copy number, size, and origin of replication, on gene transfer in vesicles. The influence of each property on the production, packaging, and uptake of vesicles containing bacterial plasmids was quantified, revealing how plasmid properties modulate vesicle-mediated horizontal gene transfer. The loading of plasmids into vesicles correlates with the plasmid copy number and is influenced by characteristics that help set the number of plasmids within a cell, including size and origin of replication. Plasmid origin also has a separate impact on both vesicle loading and uptake, demonstrating that the origin of replication is a major determinant of the propensity of specific plasmids to transfer within extracellular vesicles.

IMPORTANCE Extracellular vesicle formation and exchange are common within bacterial populations. Vesicles package multiple types of biomolecules, including genetic material. The exchange of extracellular vesicles containing genetic material facilitates interspecies DNA transfer and may be a promiscuous mechanism of horizontal gene transfer. Unlike other mechanisms of horizontal gene transfer, it is unclear whether characteristics of the exchanged DNA impact the likelihood of transfer in vesicles. Here, we systematically examine the influence of plasmid copy number, size, and origin of replication on the loading of DNA into vesicles and the uptake of DNA containing vesicles by recipient cells. These results reveal how each plasmid characteristic impacts gene transfer in vesicles and contribute to a greater understanding of the importance of vesicle-mediated gene exchange in the landscape of horizontal gene transfer.

KEYWORDS horizontal gene transfer, origin of replication, outer membrane vesicles, plasmid characteristics

The microbial world engages in dynamic and promiscuous horizontal gene transfer (HGT), believed to account for up to 20% of bacterial genomes (1–3). This genetic plasticity is vital to the adaptation and evolution of microbial species. Many environmental and cellular parameters control the flow of DNA between bacterial cells (4, 5). Most importantly, the DNA itself has significant control of the capacity for its exchange between cells. For decades, research has identified and described the roles of three mechanisms of gene transfer on the movement of genetic information between

Citation Tran F, Boedicker JQ. 2019. Plasmid characteristics modulate the propensity of gene exchange in bacterial vesicles. J Bacteriol 201:e00430-18. https://doi.org/10.1128/JB.00430-18.

Editor Anke Becker, Philipps-Universität Marburg

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Received 17 July 2018
Accepted 26 December 2018
Accepted manuscript posted online 22 January 2019
Published 13 March 2019

bacterial species: conjugation, transformation, and transduction (6). More recently, a fourth mechanism of gene transfer was identified in which genetic material is exchanged through extracellular vesicles (7–9). Although gene transfer and its limitations have been detailed for the mechanisms of conjugation, transformation, and transduction, no previous study has systematically examined how the propensity of gene transfer in vesicles depends on the properties of the exchanged DNA. Understanding the role of DNA characteristics in controlling the loading and exchange of DNA in extracellular vesicles (EVs) will explicate the contribution of vesicle-mediated gene exchange to gene flow within microbial populations.

Gene exchange in extracellular vesicles appears to be a universal route of gene transfer. A large consortium of bacterial species currently studied, from Gram negative to Gram positive, release functional extracellular vesicles (10, 11), and several species have been shown to take up genes from vesicles (12). These vesicles often contain genetic material (8, 9, 13). EVs offer a protective packaging system for DNA and other macromolecules found inside EVs, including active proteins, lipids, nucleic acids, and metabolites (14), suggesting that vesicles permit DNA transfer over longer distances and time frames. The repertoire of genetic material found in vesicles includes RNA, linear DNA fragments, and plasmid DNA, with sizes of up to 3,700 kb detected. DNA found in EVs originates from bacterial chromosomes, plasmids, and viral infection. However, no studies to date have examined whether DNA loading is regulated or random. Moreover, it is unclear if characteristics of the DNA cargo influence either vesicle production, the efficiency of loading DNA into vesicles, or the uptake of genetic material in vesicles.

Based on studies of gene transfer via conjugation, transformation, and transduction, we anticipate gene transfer in EVs will depend on the identity of the transferred DNA. The genetic content of a given plasmid governs not only the ability for it to be transferred but also the efficacy of transfer in the other three commonly studied modes of HGT (15–18). Conjugation is dependent on the origin of replication being compatible with the molecular machinery of conjugative transfers (19, 20). Plasmid size and copy number have also been shown to control plasmid transfer by conjugation (20–22). In transformation, DNA size has a significant effect on the uptake of free DNA from the environment (23–25). Transduction is also affected by plasmid characteristics. Viruses modify bacterial plasmids to generate effective transferrable DNA for transduction, including modulating the plasmid size and controlling plasmid copy number (26, 27). Since the recent discovery of vesicle-mediated gene transfer, we lack a similar understanding of the relationship between plasmid characteristics and DNA loading and transfer rates in vesicles. The only work to date is by Lamichhane et al., who demonstrated that plasmid size impacts its packaging into artificial vesicles via electroporation (28). This suggests that naturally formed EVs from bacterial cells would also be affected by DNA size. Here, we explore how these similar characteristics influence plasmid exchange in vesicles.

To probe these questions, we constructed a set a plasmids with various plasmid characteristics and quantified the influence of each characteristic on vesicle-mediated gene exchange. We investigated the roles of plasmid copy number (PCN), replication origins, and plasmid size in the production and exchange of extracellular vesicles containing plasmids. Our findings offer a systematic dissection of how changing DNA physiology is reflected in vesicle production, loading, and gene transfer.

RESULTS

Characterizing plasmid transfer within extracellular vesicles. Previous work, including our own, has looked at the role of vesicles in facilitating horizontal gene transfer between bacterial cells (12). To look more closely at the parameters that control the rate of HGT via extracellular vesicles, we examined how plasmid characteristics, specifically, plasmid copy number, size, and origin, influence DNA loading and gene transfer within EVs. Several reports have detailed how plasmid-containing EVs can be isolated and characterized (12, 29–31). Briefly, *Escherichia coli* donor strain MG1655

containing the plasmid of interest was grown to stationary phase. To harvest EVs, the cells were pelleted by centrifugation, the supernatants were filtered, and EVs were concentrated by ultracentrifugation and treated with DNase I to remove free DNA. With these harvested vesicles, we were able to quantify vesicle production as well as both the loading of specific genetic cargo into vesicles and gene transfer via the uptake of these vesicles by recipient cells.

Gel electrophoresis and quantitative PCR were used to quantify harvested EVs and their cargo. The amount of harvested vesicles was determined using SDS-polyacrylamide gel electrophoresis to measure the amount of the abundant outer membrane proteins OmpA, -C, and -F in harvested EVs. In previous studies, we found that the results from protein gels agreed with those from Bradford assays and lipid assays performed on vesicle solutions (12). Quantitative PCR was used to quantify the number of plasmids in the harvested vesicles. A standardized amount of vesicles, 0.001 mg, was lysed by boiling for 5 min, and quantitative PCR (qPCR) primers targeted to each plasmid were used (see Table S1 in the supplemental material). Standard curves were generated for each plasmid using concentrations of 0.001 ng, 0.01 ng, 0.1 ng, and 1 ng of purified plasmids plotted against their cycle threshold (C_T) values. Together, these two measurements enabled a comparison of the loading of different plasmids into EVs, reported as plasmids per picogram of vesicle protein. These numbers converted to between 1% and nearly 100% of the EVs containing a target plasmid (12) (see Table S2).

The ability of plasmid-containing EVs to facilitate gene exchange was measured in gene uptake experiments. As previously reported, for all EV uptake measurements, a uniform amount of EVs was used in all transfer experiments, specifically, 0.01 mg of corresponding membrane protein for harvested vesicles (12). The plasmid contains a resistance marker not found in the recipient strain. Over time, aliquots of the recipient culture were plated on agar medium plates selective for the resistance marker on the plasmid to monitor EV-mediated plasmid transfer. EVs loaded with specific plasmids have a characteristic uptake time (12). The time needed for a cell in a population to gain antibiotic resistance after the addition of harvested vesicles is referred to as the gene transfer time. Previously, we showed that the gene transfer time did not depend on the resistance marker used and was not strongly influenced by the time needed to gain resistance after gene uptake (12).

Using these two metrics, namely, plasmid loading and gene transfer time, we examined sets of plasmids with variable copy numbers, sizes, and origins to understand how plasmid characteristics influenced the rate of horizontal plasmid transfer in EVs.

Influence of plasmid copy number on EV-mediated gene transfer. The regulation and control of molecules that are shuttled into EVs during biogenesis are not understood. Given that no known machinery for loading cargo into vesicles has been identified and that previous measurements found that in some cases, only a small percentage of EVs were loaded with plasmid cargo (12), the loading of plasmids into vesicles may be a random process. If the process is random, it seems likely that the loading of plasmids into EVs and the subsequent delivery into recipient cells scale with plasmid copy number.

To test this hypothesis, we modified the replication origin of the plasmid pSC101 to manipulate the plasmid copy number (PCN), or the average number of plasmids per cell. Previous work has shown that specific point mutations in the SC101 origin have large effects on PCN (32). The three plasmids constructed were named SC101, SC101+, and SC101++ (Fig. 1A). After construction, each plasmid was electroporated into the donor *E. coli* strain. The plasmid copy number was determined as the ratio of the number of plasmids to the number of copies of the chromosomal gene *dxs* by using qPCR (33). As shown in Fig. 1A, these modifications of the origin increased the PCN from ~5 to 250.

Next, we quantified the impact of increased PCN on plasmid loading and vesicle-mediated gene exchange. Increasing the plasmid copy number of pSC101 did not

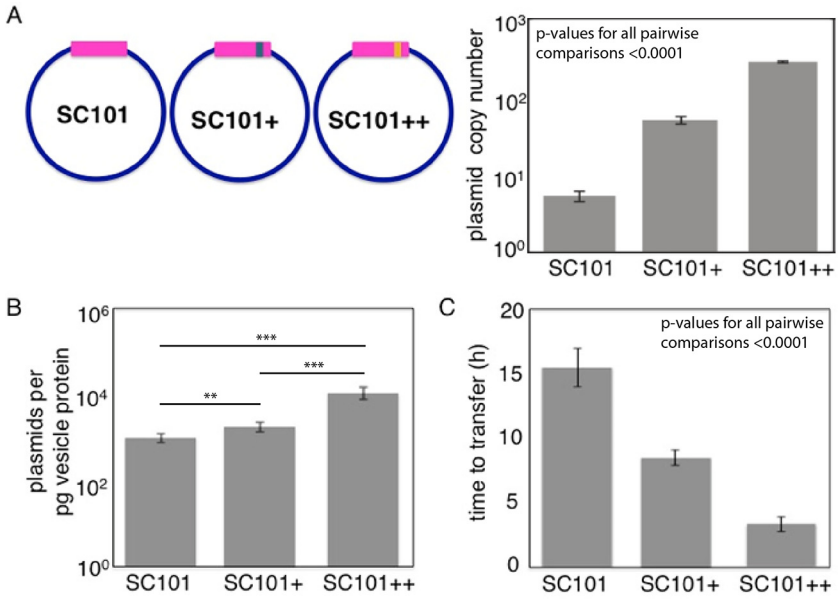

FIG 1 Tuning of plasmid copy number controls loading into vesicles. (A) Three plasmids were constructed using point mutations of the pSC101 replication origin, generating plasmids with increasing plasmid copy number, as confirmed by qPCR. (B) The number of plasmids per picogram of vesicle protein increased with increased plasmid copy number. (C) The gene transfer time decreased as plasmid copy number increased. Error bars signify standard deviations. **, $P < 0.01$; ***, $P < 0.001$.

influence the total amount of vesicles harvested from liquid culture or the average size of vesicles (see Fig. S1 and S2). We measured plasmid packaging of SC101, SC101+, and SC101++ into EVs. The number of plasmids loaded into vesicles as measured per vesicle protein increases with increasing PCN (Fig. 1B). Previously, we showed that vesicle-mediated gene transfer is dose dependent; the rate of gene transfer via EVs was proportional to the number of plasmid-containing vesicles added (12). Transfer experiments confirmed that the gene transfer time decreased as the PCN increased (Fig. 1C).

Plasmid size weakly impacts gene exchange in vesicles. In addition to copy number, we speculated that plasmid size might also influence the rate of gene exchange in vesicles. The DNA size affects the mobility of plasmids during horizontal gene transfer in conjugation and has been shown to limit transformation (34, 35). To examine the effects of plasmid size on extracellular vesicle loading and gene transfer, we generated four plasmids of various sizes based on plasmid pLC291. To increase plasmid size, we inserted nonfunctional lambda phage DNA into the plasmid using Gibson assembly, as described in Materials and Methods. The final plasmids were 3.5, 7, 10, and 15 kb in size, as verified by restriction digestion (Fig. 2A). Each plasmid was named according to its size in kilobases: pLC-3.5, pLC-7.5, pLC-10, and pLC-15. *E. coli* cells were electroporated with each plasmid, and vesicles were harvested and characterized as described above. Vesicle production slightly increased as plasmid size increased; pLC-3.5 and pLC-7.5 plasmids produced 0.48 and 0.5 mg, respectively, whereas pLC-10 and pLC-15 produced 0.7 mg and 0.76 mg, respectively (see Fig. S3). Although vesicle production was affected by plasmid size, the EV size remained unchanged. We measured vesicle size distribution by dynamic light scattering (DLS) and report consistent distributions of vesicle sizes among EVs carrying no plasmids and those packed with plasmid pLC-15 (see Fig. S4).

Next we explored the effects of plasmid size on the efficiency of DNA loading into extracellular vesicles. Using purified EVs from bacterial cell cultures carrying one of four plasmid sizes, pLC-3.5, pLC-7.5, pLC-10, and pLC-15, we performed quantitative PCR to measure plasmid copy number per picogram of vesicle protein (Fig. 2B). There is a weak trend toward greater loading for smaller plasmids. Using previously described methods, the number of plasmids per vesicle was also calculated (Table S2). Plasmids pLC-3.5 and

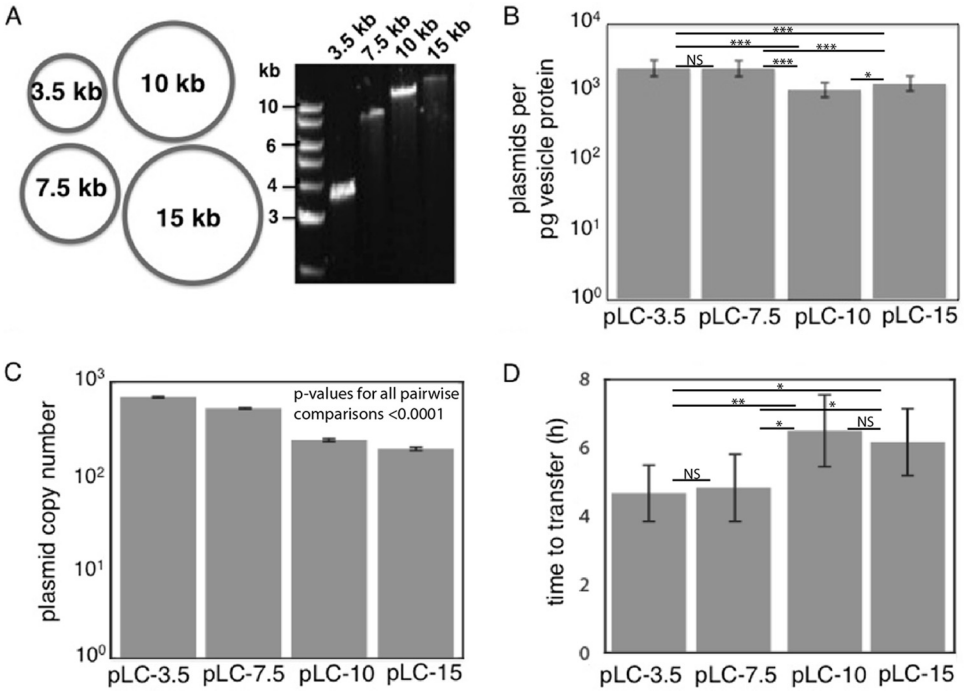

FIG 2 The impact of plasmid size on vesicle production and transfer. (A) Four plasmids were constructed with pLC291 origin and variable lengths of nonfunctional lambda phage DNA. Plasmid construct sizes were confirmed using restriction digestion and gel electrophoresis. (B) The number of plasmids per picogram of vesicle protein. (C) Plasmid copy number per genomic copy was quantified by qPCR. (D) Gene transfer time for vesicles containing plasmids pLC-3.5, pLC-7.5, pLC-10, and pLC-15. Error bars signify standard deviations. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; NS, $P > 0.05$.

pLC-7.5 had a packaging of 0.26 and 0.18 plasmid per vesicle, respectively, approximately 2 times more than the packaging of both pLC-10 and pLC-15 plasmids, which have loadings of 0.079 and 0.078 plasmid per vesicle, respectively (Table S2). To see if plasmid copy number plays a role in DNA loading, we measured plasmid copy number per genome copy of each plasmid in stationary-phase *E. coli* cells by using quantitative PCR. Plasmid copy number was inversely affected by plasmid size: 686, 519, 235, and 189 copies per genomic copy for pLC-3.5, pLC-7.5, pLC-10, and pLC-15 plasmids, respectively (Fig. 2C and Table S2). Bacterial extracellular vesicles were capable of loading a range of plasmid sizes, from 3.5 to 15 kb.

To determine if plasmid size influenced vesicle-mediated gene uptake, extracellular vesicles isolated from donor strains containing each plasmid were used in transfer experiments, as described above. Figure 2D shows a slight increase in transfer time as the plasmid gets larger. The results indicate that vesicle-mediate gene transfer is effective for a range of plasmid sizes and that plasmid packaging and transfer times were similar for plasmid sizes up to 15 kb.

Plasmid origin affects DNA loading and transfer time. In addition to plasmid copy number and size, origin is another characteristic of a plasmid that may play a role in horizontal gene transfer. The plasmid origin encodes the regulatory mechanism controlling plasmid replication, but the origin also contributes to multiple aspects of plasmid physiology (36, 37).

To evaluate the effects of plasmid origin on vesicle production and exchange, we constructed three plasmids for comparison, all 3.5 kb in size, with different origins: pMB1, pLC with dual origins of RK2 and ColE1, and SC101 (Fig. 3A). pMB1 has a ColE1-like origin of replication with high copy number. This origin of replication is controlled by Rom/Rop proteins priming the interaction with RNA I, leading to high copy number (38). pLC is the same as pLC-3.5 from Fig. 2, and SC101 is the lowest copy number variant of the pSC101 plasmid used in Fig. 1. The RK2 origin from pLC uses an

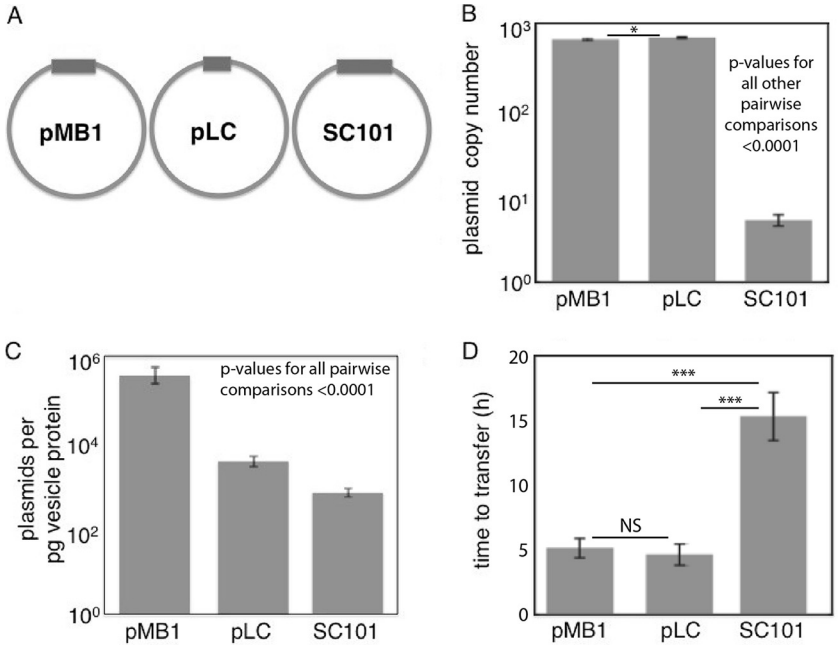

FIG 3 Impact of plasmid origin on vesicle production and size. (A) Three 3.5-kb plasmids were constructed with different origins of replication: pMB1, RK2+ColE1 (pLC), and SC101. (B) Plasmid copy number per genomic copy was quantified by qPCR. (C) Number of plasmids per picogram of vesicle protein quantified by qPCR. (D) Extracellular vesicles were isolated from *E. coli* cells carrying one of three plasmids types, pMB1, pLC or SC101, and used in gene transfer measurements. Error bars signify standard deviations. *, $P < 0.05$; ***, $P < 0.001$; NS, $P > 0.05$.

internal initiation codon of *trfA* and allows for broad-host-range maintenance (39). Both ColE1 derivatives and RK2 origins have been shown to be targeted to specific subcellular locations near midcell (39). The pSC101 origin uses a Rep initiator protein to bind intron regions controlling replication and includes a partitioning locus to stabilize inheritance (40). In our previous study, we observed that plasmids with different origins were transferred by EVs at differing rates (12), although the plasmids compared previously were of various sizes. Here, we constructed plasmids with different origins and a uniform size to examine the isolated role of the origin of replication on vesicle-mediated transfer rates. The PCNs for these plasmids were widely different, ranging from 6 to >600, as reported in Fig. 3B and Table S2. As described above, we purified vesicles from *E. coli* cells transformed with each plasmid and measured EV production using SDS-PAGE gels stained with Coomassie blue (see Fig. S5). Vesicle production and vesicle sizes were similar between EVs with different origins, with pMB1 producing slightly more vesicles (Fig. S5 and S6).

We next measured the packaging of each plasmid type (pMB1, pLC, and SC101) into vesicles. There were 364.45×10^3 copies per pg of vesicle protein of plasmid pMB1 and 3.13×10^3 and 1.12×10^3 copies per pg of vesicle protein for pLC and SC101, respectively (Fig. 3C). Plasmid copy number per vesicle was also calculated using methods for quantifying vesicle number by outer membrane protein concentration and an average vesicle diameter of 0.2 μm. *E. coli* cells grown in liquid cultures loaded 1.45 plasmids per vesicle of pMB1, 30 times more than for the SC101 plasmid (0.05 copy per vesicle), and approximately 10 times more than for RK2 (0.18 copy per EV).

Plasmid physiology can have significant control over its own range and capacity for horizontal gene transfer (34, 39, 41). To examine how plasmid origin influences gene transfer in vesicles, we performed transfer assays as described above and observed that DNA transfer was strongly affected by plasmid origin (Fig. 3D).

Role of plasmid characteristics in plasmid packaging into vesicles and subsequent rates of vesicle-mediated gene transfer. Combining the results for how

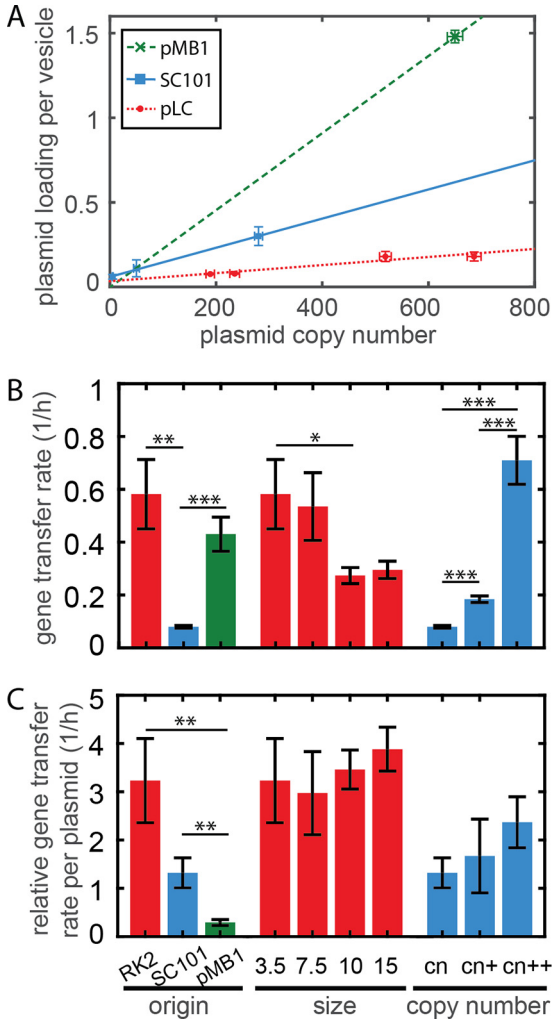

FIG 4 Summarizing the impact of plasmid characteristics on vesicle packaging and DNA transfer rates. (A) Plasmid loading per vesicle is plotted against plasmid copy number. Lines show linear fits for each plasmid origin. (B) Gene transfer rates calculated from time-to-transfer measurements. (C) Gene transfer rates normalized to the average number of plasmids per vesicle. Error bars show standard errors. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

plasmid copy number, size, and origin influence vesicle-mediated gene exchange, we see several intriguing trends. The process of gene exchange in vesicles can be separated into two essential steps, namely, the packing of genetic material into vesicles made by a donor cell and the uptake of these vesicles by a recipient cell. Figure 4A shows the packaging of all plasmids used in this study versus the copy number. Here, we used the same amount of characteristic outer membrane proteins in harvested vesicles and vesicle sizes to calculate the average loading of plasmids per vesicle, as reported in Table S2. We observed a linear increase in plasmid packaging with PCN, but only for plasmids with the same origin. Each origin seems to follow its own linear packaging curve. The strong and separate influence of origin on plasmid packaging into vesicles can clearly be seen when comparing the pLC plasmid, with a PCN of 686 and which packages 0.18 plasmid per vesicle, to pMB1, with a similar PCN of 650 and that packages 1.48 plasmids per vesicle. Although plasmid variants with RK2 and pMB1 origins have similar PCNs, pMB1 loaded nearly 10 times more plasmids into vesicles, demonstrating a significant role of the replication origin on DNA packaging in vesicles.

A comprehensive view of the influence of plasmid size, PCN, and origin on vesicle uptake is shown in Fig. 4B. Measurements of the time to transfer in gene uptake experiments were used to calculate the gene transfer rate. Gene uptake was approxi-

mated to follow a Poisson process with rate constant *r*. By using a maximum likelihood approach, we determined the value of the gene transfer rate (*r*) that was most probable given the time-to-transfer measurements for each plasmid (see supplemental text and Fig. S7). The fitting procedure also took into account a potential delay between the actual gene transfer event and the detection of the transfer event by selective plating. Figure 4B shows that gene transfer rates varied approximately an order of magnitude from 0.07 h^{-1} to 0.7 h^{-1}. As expected, plasmids with high copy numbers, such as SC101++, pLC-3.5, and pMB1, had the highest rates of gene transfer. Vesicle-mediated transfer occurs at approximately 2×10^{-20} gene transfer event per hour per vesicle per recipient cell. Although slow, this leads to transfer events within hours to days. For further discussion, see the supplemental information in our previous study (12).

For all transfer measurements, uniform amounts of vesicles were added to the donor cultures; however, as shown in Fig. 4A, the amount of plasmid per vesicle depended on both the origin and PCN. A different perspective emerges when the uptake rate is normalized for the average number of plasmids per vesicle. As shown in Fig. 4C, on a per plasmid basis, the gene transfer rate was not strongly influenced by plasmid copy number or size. Unpaired *t* tests of gene transfer rates per plasmid between plasmids with the same origin had *P* values of >0.12, with most pairs having a *P* value >0.5. Plasmids with a higher copy number had a greater chance of being incorporated into a vesicle, but all vesicles containing plasmids with the same origin were equally likely to be taken up. Instead, the origin was the major determinant of gene uptake in vesicles. Vesicles containing pLC plasmids had a roughly 10-fold-greater rate of uptake than vesicles containing a pMB1 plasmid. Together, these plots show that plasmid copy number, size, and origin, through its influence on copy number, impact the packaging efficiency of plasmids into vesicles. Plasmid origin also has an important secondary influence on the vesicle uptake rate.

DISCUSSION

Recent reports have demonstrated that extracellular vesicles mediate gene exchange within bacterial populations (9, 12, 42), but unlike other mechanism of HGT, no study has systematically examined how gene exchange rates in vesicles depend on characteristics of the exchanged genetic material. DNA characteristics were previously shown to influence the efficacy and rate of DNA exchange in other mechanisms of HGT (26, 27, 35). Our study delineates plasmid characteristics, including plasmid copy number, plasmid size, and the origin of replication, to understand individual DNA characteristic contributions to DNA loading and vesicle-mediated gene transfer. Our results demonstrate that vesicles direct the exchange of diverse genetic material and that several characteristics of the exchanged genetic elements modulate transfer.

In our previous study, we showed that the time of transfer in vesicle-mediated exchange is scaled to increasing doses of EVs (12). Here, we demonstrate that the dose-dependent control on transfer time is directly regulated by the plasmid copy number. The higher the PCN, the more plasmid is loaded and the faster the transfer time. The increased efficiency of vesicle-mediated gene exchange with higher PCNs suggests a selective pressure toward higher copy number for plasmids that rely on vesicle-mediated exchange for maintenance in a bacterial population. It has been shown that in some circumstances, an increased plasmid copy number can burden the cell, which results in a loss of plasmids from the cell at a specific threshold (43, 44). Therefore, we speculate the evolution of some plasmids favors a copy number that balances both plasmid maintenance and effective transfer between cells.

We also examined the influence of size on vesicle-mediated HGT. Our data show that plasmids of up to 15 kb are transferred effectively to recipient cells with minimal time delay compared to that of a plasmid size of 3.5 kb (Fig. 2D). *E. coli* cells are capable of loading a broad spectrum of plasmid sizes into extracellular vesicles. Plasmid size appears to indirectly influence packaging into vesicles through its effect on copy number (Fig. 2B; see Table S2 in the supplemental material). Bacteria may favor smaller

plasmid sizes that are more proficient in DNA transfer, although more work is needed to directly connect plasmid properties with gene transfer in wild bacterial populations.

Origins of replication also control plasmid exchange in vesicles. Plasmid replicons play important roles in plasmid physiology. Origins control copy number and replication efficiency and ensure maintenance. Plasmid origins also affect host ranges across bacterial species. Here, we demonstrate that plasmid origin has large effects on both the loading and uptake of plasmids in vesicles (Fig. 3). Although DNA loading scales with plasmid copy number, linear scaling only holds for plasmids with the same origin (Fig. 4A). The mechanism through which vesicle loading is biased for some plasmid origins remains unclear. The origin is known to influence plasmid localization in the cell, which can affect mobility during conjugation and transformation (36, 37). DNA topology also depends on the origin of replication (45, 46), which may have an effect on the ability to load into vesicles.

The replication origin also controls the rates of plasmid transfer through a mechanism independent from vesicle loading. Vesicles containing variants of the pLC plasmids were successfully taken up 11 times faster than those containing pMB1 (Fig. 4B and C). The mechanism through which origin regulates plasmid uptake in vesicles is unclear. The contents of a vesicle might influence the characteristics of the vesicle membrane, such as the molecular composition, shape, or charge of the vesicle. Such changes to the outside the membrane would likely influence uptake. Another possibility is that the origin influences the distribution of the number of plasmids per vesicle. Here, uptake times were normalized according to the average number of plasmids per vesicle. The loading of many plasmids into a single vesicle should reduce the uptake rate per plasmid. Given that plasmids of a length of 11,000 bp are known to have a radius of gyration near 170 nm (47), it seems unlikely that more than a few plasmids would fit into each vesicle. Supercoiling, DNA binding proteins, and processes such as plasmid dimerization, or handcuffing (48), that occur for some plasmids should influence the plasmid distribution among vesicles. The success rate of gene uptake might be a third mechanism through which origin impacts vesicle-mediated gene uptake. Although the biophysics of transferring vesicle cargo into a recipient cell are not yet resolved, gene uptake in vesicles likely involves fusion of the vesicle with the recipient membrane and movement of the genetic material from the vesicle lumen to the cytosol of the recipient cell (49, 50). The origin potentially controls the efficiency of the second step, with some origins leading to plasmids "getting lost" before uptake of the plasmid is complete. These possible ways in which plasmid origin influences vesicle-mediated gene uptake are speculative at this point, and mechanistic studies of the process of both vesicle uptake in general and gene uptake through vesicles are needed.

These results, for the first time, quantify the relationship between plasmid characteristics and vesicle-mediated gene transfer. Vesicle-mediated transfer offers a new possibility for the exchange of larger-size plasmids than by transformation. We demonstrate that loading into vesicles scales linearly with PCN, but only for plasmids with the same origin. Loading of low-copy-number plasmids into vesicles is 10% or less, which supports a random or, at the very least, inefficient loading mechanism. Vesicle-mediated exchange may be most relevant for the movement of high-copy-number plasmids. The second major conclusion is that plasmid origin is a major factor that determines the efficiency of exchange in vesicles. The impact of origin on PCN and its independent contribution to vesicle-mediated gene uptake suggest that some origins may have evolved to efficiently be transferred via vesicles. Future studies that further elucidate the mechanisms that modulate gene transfer in vesicles should help contextualize the contribution of vesicle-mediated exchange in the overall picture of horizontal gene transfer in bacterial populations.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* lab strain MG1655 was used for all extracellular vesicle and transfer experiments. Chemically competent *E. coli* 5-alpha was used for cloning (New

England BioLabs, Ipswich, MA). Bacteria were grown in Luria-Bertani (LB) broth (Difco, Sparks, MD) at 37°C with shaking at 200 rpm. *E. coli* was transformed by electroporation with plasmids listed in Table S2 in the supplemental material. Following transformation, *E. coli* was grown on LB agar plates containing either 50 $\mu g \cdot ml^{-1}$ kanamycin for SC101 plasmids and pLC291 size plasmids or 50 $\mu g \cdot ml^{-1}$ carbenicillin for pMB1. Plasmids were maintained in liquid culture with the appropriate antibiotic.

Construction of plasmids. To construct SC101 plasmids with increased plasmid copy numbers, pSC101 was used as the starting plasmid. Based on the results of Peterson and Phillips (32), a 6-bp change was made to construct plasmids pJPA12 and pJPA13 as in the aforementioned paper. Briefly, using NEB Q5 site-directed mutagenesis, GAG ATT was changed to AAG ATC or CGG ATC, respectively (New England BioLabs, Ipswich, MA).

To construct plasmids of various sizes, pLC291, 7,506 bp in length (Addgene 44448), was used as the backbone. All constructs were made via PCR amplification using Q5 DNA polymerase followed by NEB DNA assembly and 42°C heat shock transformation into chemically competent 5-alpha cells (New England BioLabs, Ipswich, MA). A 3.5-kb plasmid was constructed from regions of pLC291 that contained the origin of replication and antibiotic resistance from the deposited DNA sequences on Addgene of pLC291; these included three regions: nucleotides (nt) 900 to 1850, 2750 to 5000, and 7200 to 7500. For pLC-10 and pLC-15 (10 kb and 15 kb, respectively), we started with pLC291 and cloned in lambda DNA from VWR (Radnor, PA) using primers listed in Table S1. Confirmation of plasmid size by restriction digestion was performed on 50 ng of purified plasmids with restriction enzyme NotI at 37°C for 1 h and run on a 1% agarose gel (New England BioLabs, Ipswich, MA).

Plasmid pMB1 was constructed from pUC19 as the backbone sequence using Q5 polymerase and DNA assembly (New England BioLabs, Ipswich, MA). A nonfunctional segment of *lacZ*-harboring DNA from *E. coli* MG1655 was added to pUC19 to increase the plasmid size to 3.5 kb. All plasmids were confirmed by sequencing.

Isolation and purification of EVs. EVs were isolated from liquid cultures of *E. coli* MG1655 as previously described (51) with some modifications. Four hundred microliters of overnight culture was used to inoculate 400 ml of LB broth containing selective antibiotic. Liquid cultures were grown at 37°C with shaking at 200 rpm for 16 to 20 h. Cells were pelleted by centrifugation at 1,200 $\times$ *g* at 4°C for 30 min. The supernatants were decanted and vacuum filtrated through an ExpressPlus 0.22-μm-pore-size polyethersulfone (PES) bottle-top filter (Millipore, Billerica, MA) to remove the remaining cells and cellular debris. Vesicles were collected by ultracentrifugation at 50,000 $\times$ *g* (Ti 45 rotor; Beckman Instruments, Inc., Fullerton, CA) at 4°C for 1.5 to 2 h followed by 150,000 $\times$ *g* (Ti 70i rotor; Beckman Instruments, Inc., Fullerton, CA) at 4°C for 1.5 to 2 h, resuspended in 1 ml of phosphate-buffered saline (PBS), and stored at 4°C. Vesicle preparations were treated with 100 $ng \cdot ml^{-1}$ of DNase I at 37°C for 20 min followed by deactivation of the DNase at 80°C for 10 min. EVs with and without DNase I treatment showed similar C_T values by qPCR (see Fig. S8). Vesicle preparations were also plated on LB agar to check for the presence of bacterial cells.

EV protein concentration. Vesicle concentrations were quantified using SDS-polyacrylamide gel electrophoresis. Vesicle preparations were treated with 6$\times$ SDS loading buffer, boiled for 10 min at 100°C, run on a 10% SDS-PAGE gel (Bio-Rad Laboratories, Hercules, CA), stained for 15 min with Coomassie brilliant blue stain, and destained in H_2O, methanol, and acetic acid (50:40:10 [vol/vol/vol]) overnight. Protein concentrations were determined using ImageJ from a standard curve generated by a bovine serum albumin (BSA) protein concentration gradient, as shown in Fig. S1.

EV size characterization using dynamic light scattering. DLS was used to characterize the sizes of purified EVs. Purified EVs were analyzed using a DynaPro Titan (Wyatt Technology Corp., Santa Barbara, CA) equipped with a 0- to 50-mW laser at 830 nm as a light source. The scattered photons were detected at 90°.

Real-time PCR. DNA concentrations in purified EVs were determined using real-time PCR on a DNA Engine Opticon 2 system (Bio-Rad Laboratories, Hercules, CA) with SYBR green (Thermo Fisher Scientific Inc., Waltham, MA). Briefly, the reaction mixtures consisted of 2 μl of EVs, 0.2 μM primers, and 1 U of Phusion high-fidelity DNA polymerase (New England BioLabs Inc., Ipswich, MA) in a final volume of 45 μl. EVs were lysed by boiling at 100°C for 10 m. The program consisted of 35 cycles of denaturing at 98°C for 10 s, annealing at 60°C for 20 s, and extension at 72°C for 15 s. qPCR primers are listed in Table S1. A standard curve was generated using defined concentrations of purified plasmid: 0.001 ng, 0.01 ng, 0.1 ng, and 1 ng.

EV-mediated gene transfer. Gene transfer experiments were modified from previously published work (52, 53). The *E. coli* recipient strain was grown in 4 ml LB broth (Difco, Sparks, MD) at 37°C with shaking at 200 rpm for ~1 to 2 h to early log phase at an optical density at 600 nm (OD_{600}) of 0.2. Then at time zero, 0.01 mg purified vesicles was added. Every hour, 200 μl of culture was removed and plated on LB agar plates containing either 50 $\mu g \cdot ml^{-1}$ kanamycin or 50 $\mu g \cdot ml^{-1}$ carbenicillin, depending on the plasmid resistance. After 16 h of incubation at 37°C, CFU were counted and scored. The bacterial colonies that acquired antibiotic resistance were reselected on antibiotic selection plates, and the presence of the transferred plasmid was verified for several colonies by using PCR. Gain of resistance not associated with plasmid transfer was not observed.

Statistical analysis. All two-tailed *P* values were obtained using unpaired *t* tests to compare the means with standard deviations of two groups with an *n* of >3 (see Fig. S9 and S10).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00430-18.

SUPPLEMENTAL FILE 1, PDF file, 1.1 MB.

ACKNOWLEDGMENTS

We thank Vadim Cherezov and Ming-Yue Lee for experimental assistance.

We declare no competing interests.

This work was funded by the NSF (award number MCB-1818341).

REFERENCES

1. Wolska KI. 2003. Horizontal DNA transfer between bacteria in the environment. Acta Microbiol Pol 52:233–243.
2. Arber W. 2014. Horizontal gene transfer among bacteria and its role in biological evolution. Life (Basel) 4:217–224. https://doi.org/10.3390/life4020217.
3. Vos M, Hesselman MC, Te Beek TA, van Passel MWJ, Eyre-Walker A. 2015. Rates of lateral gene transfer in prokaryotes: high but why? Trends Microbiol 23:598–605. https://doi.org/10.1016/j.tim.2015.07.006.
4. Darmon E, Leach DRF. 2014. Bacterial genome instability. Microbiol Mol Biol Rev 78:1–39. https://doi.org/10.1128/MMBR.00035-13.
5. Dimitriu T, Misevic D, Lindner AB, Taddei F. 2015. Mobile genetic elements are involved in bacterial sociality. Mob Genet Elements 5:7–11. https://doi.org/10.1080/2159256X.2015.1006110.
6. Thomas CM, Nielsen KM. 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat Rev Microbiol 3:711–721. https://doi.org/10.1038/nrmicro1234.
7. Dorward DW, Garon CF. 1990. DNA is packaged within membrane-derived vesicles of Gram-negative but not Gram-positive bacteria. Appl Environ Microbiol 56:1960–1962.
8. Yaron S, Kolling GL, Simon L, Matthews KR. 2000. Vesicle-mediated transfer of virulence genes from *Escherichia coli* O157:H7 to other enteric bacteria. Appl Environ Microbiol 66:4414–4420.
9. Klieve AV, Yokoyama MT, Forster RJ, Ouwerkerk D, Bain PA, Mawhinney EL. 2005. Naturally occurring DNA transfer system associated with membrane vesicles in cellulolytic *Ruminococcus* spp. of ruminal origin. Appl Environ Microbiol 71:4248–4253. https://doi.org/10.1128/AEM.71.8.4248-4253.2005.
10. Brown L, Wolf JM, Prados-Rosales R, Casadevall A. 2015. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. Nat Rev Microbiol 13:620–630. https://doi.org/10.1038/nrmicro3480.
11. Deatherage BL, Cookson BT. 2012. Membrane vesicle release in bacteria, eukaryotes, and archaea: a conserved yet underappreciated aspect of microbial life. Infect Immun 80:1948–1957. https://doi.org/10.1128/IAI.06014-11.
12. Tran F, Boedicker JQ. 2017. Genetic cargo and bacterial species set the rate of vesicle-mediated horizontal gene transfer. Sci Rep 7:8813. https://doi.org/10.1038/s41598-017-07447-7.
13. Renelli M, Matias V, Lo RY, Beveridge TJ. 2004. DNA-containing membrane vesicles of *Pseudomonas aeruginosa* PAO1 and their genetic transformation potential. Microbiology 150:2161–2169. https://doi.org/10.1099/mic.0.26841-0.
14. Alves NJ, Turner KB, Medintz IL, Walper SA. 2016. Protecting enzymatic function through directed packaging into bacterial outer membrane vesicles. Sci Rep 6:24866. https://doi.org/10.1038/srep24866.
15. Yin W, Xiang P, Li Q. 2005. Investigations of the effect of DNA size in transient transfection assay using dual luciferase system. Anal Biochem 346:289–294. https://doi.org/10.1016/j.ab.2005.08.029.
16. Ray JL, Nielsen KM. 2005. Experimental methods for assaying natural transformation and inferring horizontal gene transfer. Methods Enzymol 395:491–520. https://doi.org/10.1016/S0076-6879(05)95026-X.
17. Grohmann E, Muth G, Espinosa M. 2003. Conjugative plasmid transfer in Gram-positive bacteria. Microbiol Mol Biol Rev 67:277–301.
18. Seitz P, Blokesch M. 2013. Cues and regulatory pathways involved in natural competence and transformation in pathogenic and environmental Gram-negative bacteria. FEMS Microbiol Rev 37:336–363. https://doi.org/10.1111/j.1574-6976.2012.00353.x.
19. Frost LS, Ippen-Ihler K, Skurray RA. 1994. Analysis of the sequence and gene products of the transfer region of the F sex factor. Microbiol Rev 58:162–210.
20. Llosa M, Gomis-Rüth FX, Coll M, de la Cruz F. 2002. Bacterial conjugation: a two-step mechanism for DNA transport. Mol Microbiol 45:1–8.
21. Lorenzo-Díaz F, Fernández-López C, Lurz R, Bravo A, Espinosa M. 2017. Crosstalk between vertical and horizontal gene transfer: plasmid replication control by a conjugative relaxase. Nucleic Acids Res 45:7774–7785. https://doi.org/10.1093/nar/gkx450.
22. Lorenz MG, Wackernagel W. 1994. Bacterial gene transfer by natural genetic transformation in the environment. Microbiol Rev 58:563–602.
23. Claverys J-P, Martin B. 2003. Bacterial "competence" genes: signatures of active transformation, or only remnants? Trends Microbiol 11:161–165.
24. Overballe-Petersen S, Harms K, Orlando LAA, Mayar JVM, Rasmussen S, Dahl TW, Rosing MT, Poole AM, Sicheritz-Ponten T, Brunak S, Inselmann S, de Vries J, Wackernagel W, Pybus OG, Nielsen R, Johnsen PJ, Nielsen KM, Willerslev E. 2013. Bacterial natural transformation by highly fragmented and damaged DNA. Proc Natl Acad Sci U S A 110:19860–19865. https://doi.org/10.1073/pnas.1315278110.
25. Ohse M, Takahashi K, Kadowaki Y, Kusaoke H. 1995. Effects of plasmid DNA sizes and several other factors on transformation of *Bacillus subtilis* ISW1214 with plasmid DNA by electroporation. Biosci Biotechnol Biochem 59:1433–1437. https://doi.org/10.1271/bbb.59.1433.
26. Mašlaňová I, Doškař J, Varga M, Kuntová L, Mužík J, Malúšková D, Růžičková V, Pantůček R. 2013. Bacteriophages of *Staphylococcus aureus* efficiently package various bacterial genes and mobile genetic elements including SCC *mec* with different frequencies. Environ Microbiol Rep 5:66–73. https://doi.org/10.1111/j.1758-2229.2012.00378.x.
27. Clewell DB, Weaver KE, Dunny GM, Coque TM, Francia MV, Hayes F. 2014. Extrachromosomal and mobile elements in enterococci: transmission, maintenance, and epidemiology. *In* Gilmore MS, Clewell DB, Ike Y, Shankar N (ed), Enterococci: from commensals to leading causes of drug resistant infection. Massachusetts Eye and Ear Infirmary, Boston, MA. https://www.ncbi.nlm.nih.gov/books/NBK190430/.
28. Lamichhane TN, Raiker RS, Jay SM. 2015. Exogenous DNA loading into extracellular vesicles via electroporation is size-dependent and enables limited gene delivery. Mol Pharm 12:3650–3657. https://doi.org/10.1021/acs.molpharmaceut.5b00364.
29. Fulsundar S, Harms K, Flaten GE, Johnsen PJ, Chopade BA, Nielsen KM. 2014. Gene transfer potential of outer membrane vesicles of *Acinetobacter baylyi* and effects of stress on vesiculation. Appl Environ Microbiol 80:3469–3483. https://doi.org/10.1128/AEM.04248-13.
30. Ho M-H, Chen C-H, Goodwin JS, Wang B-Y, Xie H. 2015. Functional advantages of *Porphyromonas gingivalis* vesicles. PLoS One 10:e0123448. https://doi.org/10.1371/journal.pone.0123448.
31. Cai J, Wu G, Jose PA, Zeng C. 2016. Functional transferred DNA within extracellular vesicles. Exp Cell Res 349:179–183. https://doi.org/10.1016/j.yexcr.2016.10.012.
32. Peterson J, Phillips GJ. 2008. New pSC101-derivative cloning vectors with elevated copy numbers. Plasmid 59:193–201. https://doi.org/10.1016/j.plasmid.2008.01.004.
33. Lee C, Kim J, Shin SG, Hwang S. 2006. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. J Biotechnol 123:273–280. https://doi.org/10.1016/j.jbiotec.2005.11.014.
34. Smillie C, Garcillán-Barcia MP, Francia MV, Rocha EPC, de la Cruz F. 2010. Mobility of plasmids. Microbiol Mol Biol Rev 74:434–452. https://doi.org/10.1128/MMBR.00020-10.
35. Kung SH, Retchless AC, Kwan JY, Almeida RPP. 2013. Effects of DNA size on transformation and recombination efficiencies in *Xylella fastidiosa*. Appl Environ Microbiol 79:1712–1717. https://doi.org/10.1128/AEM.03525-12.
36. Bingle LE, Thomas CM. 2001. Regulatory circuits for plasmid survival. Curr Opin Microbiol 4:194–200.
37. Wegrzyn G, Wegrzyn A. 2002. Stress responses and replication of plasmids in bacterial cells. Microb Cell Fact 1:2.
38. Camps M. 2010. Modulation of ColE1-like plasmid replication for recombinant gene expression. Recent Pat DNA Gene Seq 4:58–73.
39. Pogliano J, Ho TQ, Zhong Z, Helinski DR. 2001. Multicopy plasmids are clustered and localized in *Escherichia coli*. Proc Natl Acad Sci U S A 98:4486–4491. https://doi.org/10.1073/pnas.081075798.
40. Thompson MG, Sedaghatian N, Barajas JF, Wehrs M, Bailey CB, Kaplan N,

Hillson NJ, Mukhopadhyay A, Keasling JD. 2018. Isolation and characterization of novel mutations in the pSC101 origin that increase copy number. Sci Rep 8:1590. https://doi.org/10.1038/s41598-018-20016-w.
41. Haase J, Lurz R, Grahn AM, Bamford DH, Lanka E. 1995. Bacterial conjugation mediated by plasmid RP4: RSF1010 mobilization, donor-specific phage propagation, and pilus production require the same Tra2 core components of a proposed DNA transport complex. J Bacteriol 177:4779–4791.
42. Blesa A, Berenguer J. 2015. Contribution of vesicle-protected extracellular DNA to horizontal gene transfer in *Thermus* spp. Int Microbiol 18:177–187. https://doi.org/10.2436/20.1501.01.248.
43. Slater FR, Bailey MJ, Tett AJ, Turner SL. 2008. Progress towards understanding the fate of plasmids in bacterial communities. FEMS Microbiol Ecol 66:3–13. https://doi.org/10.1111/j.1574-6941.2008.00505.x.
44. Watve MM, Dahanukar N, Watve MG. 2010. Sociobiological control of plasmid copy number in bacteria. PLoS One 5:e9328. https://doi.org/10.1371/journal.pone.0009328.
45. Rampakakis E, Gkogkas C, Di Paola D, Zannis-Hadjopoulos M. 2010. Replication initiation and DNA topology: the twisted life of the origin. J Cell Biochem 110:35–43. https://doi.org/10.1002/jcb.22557.
46. Higgins NP, Vologodskii AV. 2015. Topological behavior of plasmid DNA. Microbiol Spectr 3:PLAS-0036-201. https://doi.org/10.1128/microbiolspec.PLAS-0036-2014.
47. Robertson RM, Laib S, Smith DE. 2006. Diffusion of isolated DNA molecules: dependence on length and topology. Proc Natl Acad Sci U S A 103:7310–7314. https://doi.org/10.1073/pnas.0601903103.
48. Park K, Han E, Paulsson J, Chattoraj DK. 2001. Origin pairing ('handcuffing') as a mode of negative control of P1 plasmid copy number. EMBO J 20:7323–7332. https://doi.org/10.1093/emboj/20.24.7323.
49. Fulsundar S, Kulkarni HM, Jagannadham MV, Nair R, Keerthi S, Sant P, Pardesi K, Bellare J, Chopade BA. 2015. Molecular characterization of outer membrane vesicles released from *Acinetobacter radioresistens* and their potential roles in pathogenesis. Microb Pathog 83–84:12–22. https://doi.org/10.1016/j.micpath.2015.04.005.
50. Tashiro Y, Hasegawa Y, Shintani M, Takaki K, Ohkuma M, Kimbara K, Futamata H. 2017. Interaction of bacterial membrane vesicles with specific species and their potential for delivery to target cells. Front Microbiol 8:571. https://doi.org/10.3389/fmicb.2017.00571.
51. Klimentová J, Stulík J. 2015. Methods of isolation and purification of outer membrane vesicles from Gram-negative bacteria. Microbiol Res 170:1–9. https://doi.org/10.1016/j.micres.2014.09.006.
52. Jiang SC, Paul JH. 1998. Gene transfer by transduction in the marine environment. Appl Environ Microbiol 64:2780–2787.
53. Domingues S, Harms K, Fricke WF, Johnsen PJ, da Silva GJ, Nielsen KM. 2012. Natural transformation facilitates transfer of transposons, integrons and gene cassettes between bacterial species. PLoS Pathog 8:e1002837. https://doi.org/10.1371/journal.ppat.1002837.