AMERICAN SOCIETY FOR MICROBIOLOGY | Applied and Environmental Microbiology®

EVOLUTIONARY AND GENOMIC MICROBIOLOGY

Membrane-Binding Biomolecules Influence the Rate of Vesicle Exchange between Bacteria

Frances Tran,[a] Manasi S. Gangan,[b] Brian P. Weaver,[b] James Q. Boedicker[a,b]

[a]University of Southern California, Department of Biological Sciences, Los Angeles, California, USA
[b]University of Southern California, Department of Physics and Astronomy, Los Angeles, California, USA

Frances Tran and Manasi S. Gangan contributed equally to this article. Author order was determined in order of increasing seniority.

ABSTRACT The exchange of bacterial extracellular vesicles facilitates molecular exchange between cells, including the horizontal transfer of genetic material. Given the implications of such transfer events on cell physiology and adaptation, some bacterial cells have likely evolved mechanisms to regulate vesicle exchange. Past work has identified mechanisms that influence the formation of extracellular vesicles, including the production of small molecules that modulate membrane structure; however, whether these mechanisms also modulate vesicle uptake and have an overall impact on the rate of vesicle exchange is unknown. Here, we show that membrane-binding molecules produced by microbes influence both the formation and uptake of extracellular vesicles and have the overall impact of increasing the vesicle exchange rate within a bacterial coculture. In effect, production of compounds that increase vesicle exchange rates encourage gene exchange between neighboring cells. The ability of several membrane-binding compounds to increase vesicle exchange was demonstrated. Three of these compounds, nisin, colistin, and polymyxin B, are antimicrobial peptides added at sub-inhibitory concentrations. These results suggest that a potential function of exogenous compounds that bind to membranes may be the regulation of vesicle exchange between cells.

IMPORTANCE The exchange of bacterial extracellular vesicles is one route of gene transfer between bacteria, although it was unclear if bacteria developed strategies to modulate the rate of gene transfer within vesicles. In eukaryotes, there are many examples of specialized molecules that have evolved to facilitate the production, loading, and uptake of vesicles. Recent work with bacteria has shown that some small molecules influence membrane curvature and induce vesicle formation. Here, we show that similar compounds facilitate vesicle uptake, thereby increasing the overall rate of vesicle exchange within bacterial populations. The addition of membrane-binding compounds, several of them antibiotics at subinhibitory concentrations, to a bacterial coculture increased the rate of horizontal gene transfer via vesicle exchange.

KEYWORDS horizontal gene transfer, membrane biophysics, vesicles

Many biomolecules are exchanged via bacterial extracellular vesicles. Bacterial vesicles are known to contain cytoplasmic and membrane proteins, genetic material, and small molecules, including bacterial signaling molecules. The uptake of vesicles enables molecular transfer between different species of bacteria and from bacteria to eukaryotic host cells (1 to 6). Vesicle exchange contributes to horizontal gene transfer within bacterial populations (7 to 10). Although many mechanisms have been shown to contribute to bacterial vesicle formation (11 to 19), less is known about mechanisms cells use to control the exchange of vesicles, which involves both the production of vesicles by a donor cell and the uptake of vesicles by a recipient cell. Given

Editor Pablo Ivan Nikel, Novo Nordisk Foundation Center for Biosustainability

Address correspondence to James Q. Boedicker, boedicke@usc.edu.
The authors declare no conflict of interest.

Received 9 August 2022
Accepted 12 September 2022
Published 7 November 2022

the importance of vesicle exchange to many cellular processes and the ubiquity of vesicle production by many bacterial species (20, 21), it seems likely that bacteria would have evolved strategies to elicit and control vesicle exchange.

More is known about regulation of vesicle exchange within eukaryotic systems. Eukaryotic vesicles are essential to signal transmission within neuronal synapses and are also involved in immune regulation and angiogenesis (22 to 26). Vesicle formation and uptake both require restructuring the membrane and the formation of energetically costly intermediate states of the membrane (27 to 29). Eukaryotic cells overcome these energy barriers through the use of molecular motors and membrane-restructuring molecules to induce membrane curvature (30 to 32). Similar strategies have been shown in bacteria, with the best example being regulation of vesicle production via *Pseudomonas* quinolone signal (PQS) (11). PQS inserts into the bacterial membrane, inducing curvature and leading to increased vesicle production (11, 14, 33). PQS production can also induce vesicle formation in neighboring species (5). Other membrane-binding compounds have been shown to influence vesicle production, including polymyxin B, colistin, and phenol-soluble modulins (34 to 36). These reports show that as in eukaryotic cells, vesicle production by bacteria can be regulated by molecules that bind to and restructure the cell membrane.

It is not known if molecules that restructure the cell membrane also influence vesicle uptake by bacteria, and if the presence of such molecules impacts the overall rate of vesicle exchange within a population of bacteria. Here, we test the influence of several membrane-restructuring compounds on the rate of vesicle production and vesicle uptake to determine the extent that vesicle exchange can be controlled via exogenous compounds. Vesicle uptake was quantified through the vesicle-mediated transfer of plasmid DNA and the resulting gain of antibiotic resistance in the recipient population (10). These results demonstrate that exogenous bacterial compounds that are known to bind to and restructure the cell membrane increase vesicle exchange within bacterial populations.

RESULTS

Membrane-structuring protein alpha-synuclein increases the production and uptake of extracellular vesicles in bacteria. In eukaryotic systems, the production and uptake of vesicles is regulated by many mechanisms. One mechanism for extracellular vesicle (EV) biogenesis in eukaryotic systems includes recruitment of ESCRT (endosomal sorting complexes required for transport) complexes and their interaction with the membrane and many other factors (37, 38). As for EV uptake in eukaryotic systems, EV binding and uptake can be regulated by transmitted signals from the cell surface to elicit uptake (39). As vesicle exchange in bacterial cells could also involve restructuring and reshaping the cell membrane, we sought to determine if biomolecules known to interact with cell membrane would regulate exchange of bacterial vesicles. Initial experiments examined the influence of the well-characterized human protein alpha-synuclein (AS) on vesicle formation and uptake. AS binds to membranes and is found in high abundance in presynaptic termini associated with synaptic vesicles (40 to 42). Alpha-synuclein binds to curved, anionic lipids (43). In addition, previous studies have suggested a membranolytic effect of AS on bacterial cell (44). We speculated that the ability of AS to bind to and restructure cellular membranes would translate to modulation of vesicle production and uptake in bacteria at sublethal concentrations.

To test the ability of AS to influence vesicle production, concentrations of purified AS between 0.01 μM and 0.1 μM were added to cultures of *Escherichia coli* MG1655 containing the plasmid pLC-RK2 (10) (see Table S1 in the supplemental material). Vesicles were harvested from culture after 16 to 20 h of growth via size exclusion filtration and ultracentrifugation (see Fig. 1A). Production of vesicles was measured by quantifying the concentration of outer membrane proteins, OmpC/F, in solutions of harvested vesicles via SDS-Polyacrylamide gel electrophoresis (see Fig. S1). As shown in Fig. 1B, cultures of the *E. coli* donor strain grown in AS resulted in 2 to 3 times more vesicle production. AS at 0.1 μM did not strongly influence cell growth (see Fig. S2).

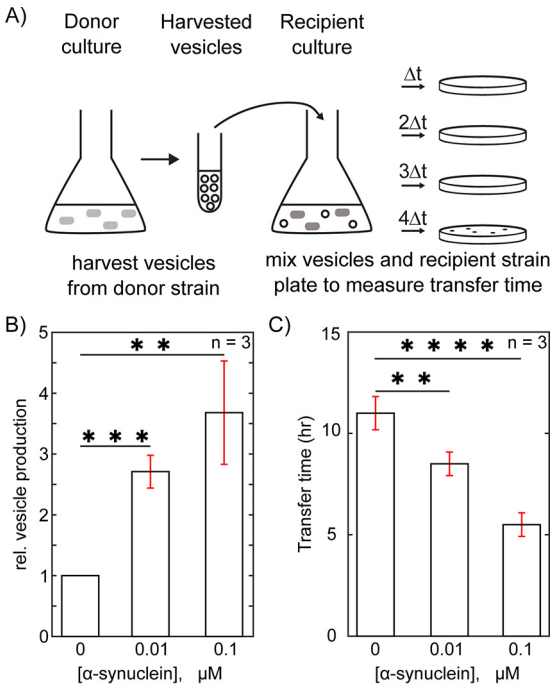

FIG 1 Alpha-synuclein increases the rates of extracellular vesicle (EV) production and uptake. (A) EVs were harvested from a donor culture via filtration and centrifugation. The donor strain contained a plasmid-conferring antibiotic resistance. Harvested EVs were added to a recipient culture, and EV uptake was monitored by detecting the gain of resistance in recipient cells. (B) Addition of the membrane binding eukaryotic peptide alpha-synuclein increased the rate of vesicle production by the *E. coli* donor strain in a dose-dependent manner. (C) Addition of alpha-synuclein to the recipient *E. coli* culture decreased the time to transfer of EVs in a dose-dependent manner. $n = 3$. Error bars show standard deviation. Significance in the difference observed in vesicle production and transfer time between treated and untreated samples was confirmed with unpaired *t* test (**, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$).

Next, we tested if these same concentrations of AS would likewise influence the uptake of vesicles by a recipient strain. The assay for vesicle uptake is depicted in Fig. 1A. Vesicles were harvested from a donor bacterial strain containing a plasmid, and the harvested vesicles, some containing the plasmid pLC-RK2, were added to a recipient bacterial strain. Aliquots of the culture of receiver strain with added harvested vesicles were removed at a set time interval and spread onto antibiotic selection plates. The plasmid contained a resistance marker, and the recipient strain did not grow on antibiotic selective plates in the absence of the plasmid. The time needed to detect a recipient cell with antibiotic resistance was defined as the time to transfer and is proportional to the rate of successful gene transfer via vesicles. In previous studies, we have shown that gene transfer in vesicles has a characteristic transfer time that depends on the concentrations and characteristics of the transferred plasmid, the donor strain, and the recipient strain (10). Gain of resistance in this assay is the result of the uptake of plasmids located inside harvested vesicles, as verified by detection of the transferred plasmid in resistant recipient strains via colony PCR. PCR using primers targeting a sequence on the transferred plasmid were performed for 5 to 10 colonies per plate, or all the colonies if the number of colonies was less than 5. Occasionally, a satellite colony was observed, and these were not tested, but no resistant colonies were detected that did not contain the transferred plasmid. Controls in which no vesicles or vesicles from a donor strain without the plasmid were added to the recipient culture were run, resulting in no resistant colonies. Vesicles from an *E. coli* MG1655 donor strain containing plasmid pLC-RK2 were added to the recipient strain, *E. coli* MG1655, at an early exponential growth phase. In transfer experiments, a standard number of vesicles was used. Vesicles added to recipient cultures contained a total of 1 μg of the outer membrane proteins OmpC/F, quantified

via protein gels (see Fig. S1). As shown in Fig. 1C, in the absence of AS gene transfer occurred after 11 h, whereas the time to transfer was shortened to 8.5 and 5.5 h after adding 0.01 μM and 0.1 μM AS, respectively.

Increased vesicle production and uptake rate in the presence of AS suggested that exogenous molecules known to bind to and restructure cellular membranes have the potential to modulate vesicle exchange between bacterial cells. Next, we tested if this phenomenon was general to other exogenous biomolecules known to interact with outer membranes, specifically compounds naturally released by bacteria.

Membrane binding exogenous molecules produced by bacteria increased vesicle production. Many molecules released by bacteria are known to bind to and restructure cellular membranes. We hypothesized that like AS, molecules naturally produced by bacteria that affect membrane structure would modulate rates of vesicle exchange. For example, *Pseudomonas* quinolone signal (PQS) has been shown to induce membrane curvature in both *Pseudomonas aeruginosa* and red blood cells and influence vesicle production (11, 14). Many other membrane-binding molecules released by bacterial cells have been characterized, including several molecules known to have antibiotic properties. Like PQS, the membrane binding antibiotic compounds colistin and polymyxin B (PMB) increased the rate of vesicle production by bacteria (35).

Here, we measured the influence of membrane-structuring molecules such as colistin, nisin, PMB, and PQS on horizontal gene transfer (HGT) via EVs, as each of these molecules is known to bind to bacterial membranes, and modulation of membrane shape has been observed (45 to 48). Among these, colistin and PMB are known inhibitors of *E. coli* growth. In our tests, concentrations below the reported MIC were used (49, 50) (see Table S2). In Table S2, we define the baseline or 1$\times$ concentration used for each compound tested. As shown in Fig. S3A, colistin and PMB at this 1$\times$ concentration had a temporary effect on cell growth, although normal growth resumed after a few hours. Colistin and PMB increased the number of cells in the population with compromised membranes, as measured using propidium iodide, but that effect was also transient, as shown in Fig. S3B and S3C. Though Toyofuku et al. showed a small decrease in *E. coli* growth upon treatment with 50 μM PQS, several studies indicate that PQS does not have reported MIC for *E. coli* cultures (51 to 53). On the other hand, nisin does not have a well-defined MIC for *E. coli*. The respective 1$\times$ concentrations were arbitrarily fixed at 10 μg/mL and 20 μg/mL (Table S2). We observed no decrease in the growth rate or loss of membrane integrity when *E. coli* cultures were treated either with nisin or with PQS, at 1$\times$ concentrations (Fig. S3). EV production and uptake were measured in the presence of each compound using the assays described in Fig. 1A. In control samples, *E. coli* were treated with 0.5 ug/mL bovine serum albumin (BSA) or treated with 1 uM N-butyryl-L-homoserine lactone (C4-AHL). Both BSA and C4-AHL are not known to bind to restructure bacterial membrane, and C4-AHL has been shown not to influence vesicle production in bacteria (54).

Vesicle production was measured by quantifying the abundance of outer membrane proteins in purified vesicle on SDS-PAGE gel. These measurements were also compared to nanoparticle tracking analysis, which directly counts EVs in solution (Fig. S4), as recent publications have shown that vesicle yields using protein-based assays can be unreliable when comparing different strains and growth conditions (55). As shown in Fig. 2A and Fig. S5, all three antibiotic compounds and the PQS positive control increased vesicle production of the *E. coli* donor strain, similar to previous reports (11, 14, 56). Vesicle production in the presence of these compounds was concentration dependent (Fig. 2B). Even upon treatment with 0.25$\times$ relative concentration, a nearly 2-fold increase in vesicle production was observed, demonstrating that even at low concentrations, far below the MIC of colistin and PMB, these compounds have the potential to influence vesicle production. Vesicle size and morphology were not strongly affected by these compounds (Fig. S6).

Bacterial-membrane-binding compounds increase vesicle uptake in recipient cells. The induction of membrane curvature is also essential for vesicle fusion and therefore vesicle uptake with recipient cells (11, 57 to 59). As shown in Fig. 1, alpha-

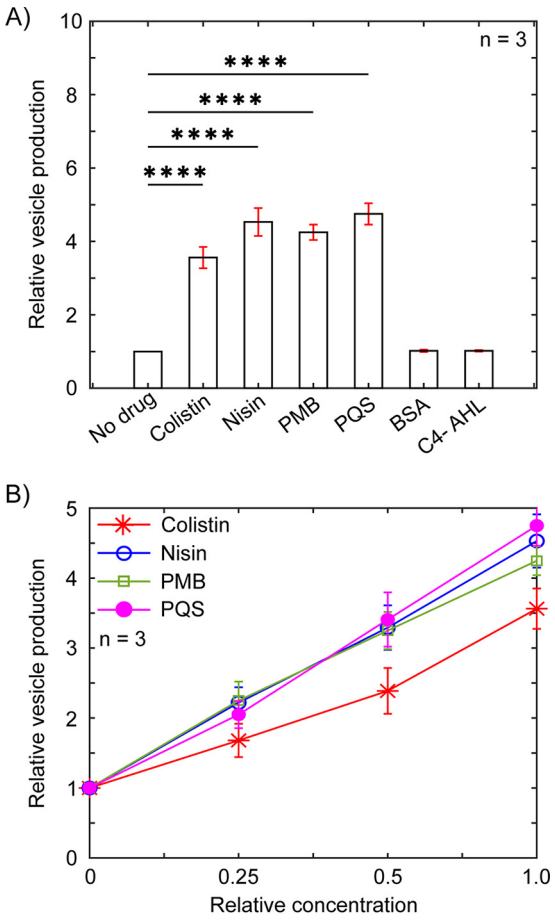

FIG 2 Membrane-binding compounds produced by bacteria increased vesicle production. EV production was measured by analyzing the concentration of characteristic outer membrane proteins (OmpC/F) in harvested EVs. (A) Addition of exogenous molecules increased EV production in a culture of *E. coli*. (B) Vesicle production increased linearly with increase in drug concentration. 1× relative concentration for colistin and PMB is 1 μg/mL and for nisin and PQS is 10 and 20 μg/mL, respectively. Error bars show standard deviation. Difference between the experimental conditions was validated with unpaired *t* test (****, $P \leq 0.0001$).

synuclein, a molecule known to restructure membranes, influenced vesicle production and uptake.

Next, we tested if the four compounds shown to induce vesicle production also increased vesicle uptake. As in Fig. 1, vesicles were harvested from a donor *E. coli* strain containing the plasmid pLC-RK2, which confers kanamycin resistance to the host cells (10). Donor cells were grown in the absence of the membrane-binding compound, although as shown in Fig. S7, EV transfer time was not dependent on whether EVs were produced in the presence or absence of membrane-binding compounds. Recipient cells grown to exponential phase were treated for 1 h with one of the membrane-binding compounds prior to the addition of harvested vesicles. Cells were plated every hour on LB plates with kanamycin to track plasmid transfer. PCR-using primers targeting a sequence on the transferred plasmid were performed for 5 to 10 colonies per plate, or for all of the colonies if the number of colonies was less than 5. Occasionally a satellite colony was observed, and these were not tested. No resistant colonies were detected that did not contain the transferred plasmid. In controls, no vesicles, or vesicles from a donor strain without the plasmid, were added to the recipient culture, resulting in no resistant colonies. Vesicles harvested from a donor containing pLC-RK2 transferred around 10 h in the absence of added compound. Uptake in the presence of the 4 membrane-binding molecules tested decreased in transfer time to 5 to 6 h (see Fig. 3A). Negative

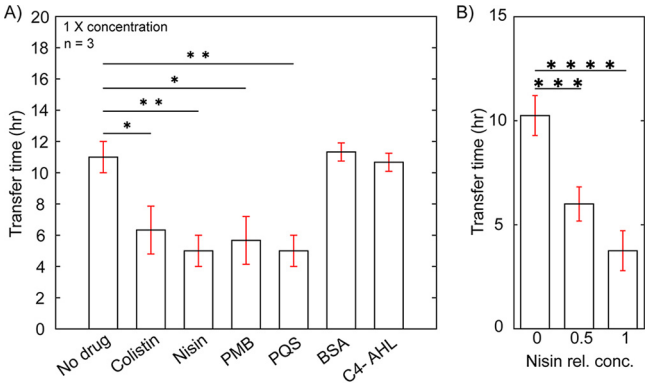

FIG 3 Membrane-binding compounds produced by bacteria increase vesicle uptake. Vesicle uptake was quantified as the time needed for recipient cells to gain antibiotic resistance as the result of plasmid transfer via EV uptake. (A) Colistin, nisin, polymyxin B(PMB), and *Pseudomonas* quinolone signal (PQS) signal all increased EV uptake in a culture of *E. coli*. Bovine serum albumin (BSA) and N-butyryl-L-Homoserine lactone (C4-AHL) were negative controls. (B) Nisin increased EV uptake in a dose-dependent manner. Error bars show standard deviation. Unpaired *t* test was used to confirm the difference between treated and untreated cultures (**, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$).

controls showed that 0.5 μg/mL BSA and 1 μM C4-AHL did not alter the transfer time of the plasmid. The reduction in the uptake time was dependent on the concentration of the added compound, as shown for the case of nisin in Fig. 3B.

Membrane-binding compounds increased the rate of horizontal gene transfer within a bacterial coculture. Given that the exogenous molecules tested increase both vesicle production and uptake rates, we next tested if the addition of these compounds would influence plasmid exchange within a bacterial coculture. As shown in Fig. 4A, exponential cultures of *E. coli* strains carrying different plasmids were mixed together. One strain was *E. coli* MG1655 carrying the pLC291 plasmid with kanamycin resistance, and the other strain was *E. coli* DH5α carrying pSC101+ plasmid with ampicillin resistance. Control experiments confirmed that the plasmids were compatible and could be stably maintained in the same cell (data not shown). We hypothesized that plasmid exchange via EVs would result in a strain with resistance to both antibiotics. Strain DH5α was chosen because its genome contains a deletion of *lacZ*, enabling discrimination of the direction of gene flow via selection on MacConkey agar plates (see Fig. S8).

After inoculating the coculture, 1 mL aliquots were removed every hour, at the passage time, and used to inoculate fresh media with double antibiotic selection. This culture, called the gene transfer screen in Fig. 4a, contained kanamycin at 50 μg/mL and ampicillin at 100 μg/mL; therefore, only cells containing both resistance markers would proliferate. The fold change in the optical density at 600 nm after 12 h in the gene transfer screen was used to determine whether plasmid exchange had occurred within the initial coculture prior to the time of cell passage. This protocol screened a larger population of cells than plating, allowing us to more reliably detect a small number of cells with double resistance. As shown in Fig. 4B, in the absence of externally added membrane binding molecules (condition Strains A+B), cells with double antibiotic resistance were detected after 9 h of coculture. For aliquots of coculture sampled prior to 9 h, the optical density of the culture in the presence of both antibiotics decreased over time, whereas coculture aliquots taken at 9 h or later resulted in an increase in optical density over time. Growth in the gene transfer screen indicated that the coculture contained cells with both plasmids at the time of passage. Cells growing within the gene transfer screen were streaked out to form single colonies on McConkey's agar plates with kanamycin and ampicillin, as shown in Fig. S9. We further confirmed the presence of pLC291 and pSC101+ plasmids in transformants using PCR and analysis of restriction digestion patterns of reisolated plasmids. PCR primers targeted bla and npt resistance genes to identify the presence of either the pLC291 or pSC101+ plasmids. In controls, PCR products were only observed in host cells containing the plasmid of

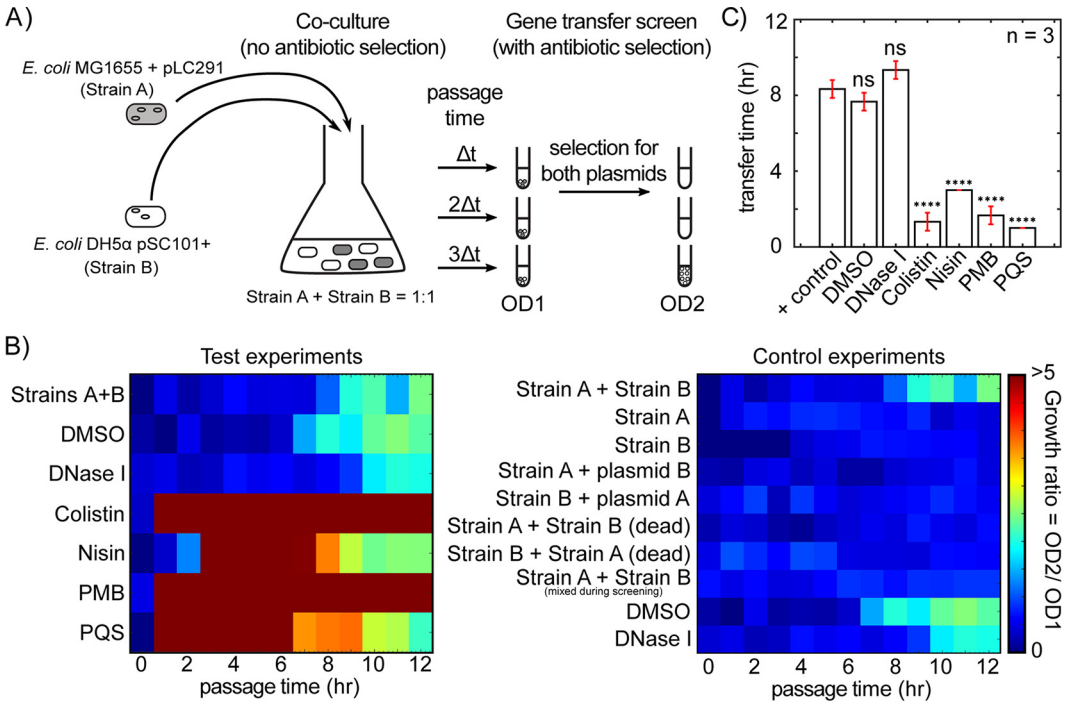

FIG 4 Membrane-binding molecules increased the rate of horizontal gene transfer within a bacterial coculture. (A) Two strains of *E. coli* harboring plasmids with different antibiotic resistance genes were cocultured. Over time, aliquots of the coculture were used to inoculate media containing both antibiotics to screen for cells containing both plasmids. OD1 is the optical density of cells at the beginning of the gene transfer screen, and OD2 is the optical density of cells growing in double antibiotic selection after 12 h. Growth within the gene transfer screen indicates plasmid exchange within the coculture prior to the passage time. (B) The change in optical density within the gene transfer screen was used to compare the rate of plasmid exchange under a variety of conditions, with Strains A + B indicating the positive control. Treatments include the addition of colistin, nisin, PMB, and PQS at the 1× concentration to the coculture. Controls include monocultures, monocultures with free plasmid, mixtures of live and dead strains, coculture in the presence of DNase I, with 0.1% vol/vol DMSO, and mixing of two monocultures during the gene transfer screen. (C) Transfer time for the positive control (strain A + strain B), compared to treatments with membrane binding compounds. Treatment with DMSO or DNase I are shown as negative controls. Transfer time is the first passage time at which plasmid transfer was detected. Error bars indicate standard deviation. Difference between the experimental conditions was validated with unpaired *t* test (****, $P \leq 0.0001$).

interest (Fig. S10). We randomly selected colonies from the McConkey's plate and confirmed the presence of both of the resistance genes using PCR (Fig. S11). Additionally, we reisolated plasmids from a culture inoculated from the transformed colony and digested it with EcoNI. DNA fragments were the same as in a digestion of a mixture of the pLC291 and pSC101+ plasmids (Fig. S12), confirming cells within the colony harbored both plasmids.

As shown in Fig. 4B and C, in the presence of membrane binding compounds, the time needed to observe a strain with double antibiotic resistance was decreased from 8 h to less than 4 h. Control experiments confirmed that (i) monocultures of cells with only one plasmid did not gain double antibiotic resistance, (ii) free plasmid added to a monoculture did not result in gene transfer, (iii) dead cells were incapable of transferring a plasmid, and (iv) genetic transfer did not happen in double selection medium. DNase I activity within the coculture also did not significantly change the time needed for gene transfer, suggesting that the transferred plasmid was protected from DNA degradation. Plasmid transfer within the coculture was also faster when treatments were added at 0.25× concentration, as shown in Fig. S13.

DISCUSSION

Vesicle exchange is critical to many bacterial processes, such as host invasion, signal exchange, and gene transfer. Vesicle exchange appears to be ubiquitous and is not known to require specialized molecular machinery for vesicle production or uptake.

It seems likely there would be selection pressure to evolve strategies to regulate vesicle exchange given its potential to facilitate horizontal gene transfer. The ability of cells to regulate rates of horizontal gene transfer has been observed previously for other mechanisms of gene transfer (60 to 64). For example, the studies carried out by the Timmis group in 1994 showed cell lysis mediated by lytic enzymes derived from λ phage, leading to the release of a large amount of extracellular DNA, which was in turn absorbed by the cells. These studies further showed that transformation scales with the availability of free DNA (65). Previous studies have revealed several biological parameters that influence vesicle production, including modulating membrane composition (13, 66), activation of stress response pathways (35, 59, 67), destruction of the cell wall, and the production of membrane structuring molecules (13, 35, 59, 66, 68, 69). It is not surprising that membrane-binding molecules would influence the production of vesicles, as eukaryotic cells utilize molecules that wedge, crowd, and bend the membrane to overcome the energetic costs of vesicle production. Here, we showed that membrane-binding molecules produced by bacteria also increased the rate of vesicle uptake. Some short peptides facilitate membrane fusion, including fusion peptides and also some antimicrobial peptides (70). Membrane fusion is promoted through a combination of induction of membrane curvature, charge screening, anchoring two membranes in juxtaposition, and even modulation of membrane rupture tension (70). It remains unclear how the bacterial peptides tested here facilitate membrane fusion.

Here, we tested the ability of four bacterial compounds, nisin, colistin, PQS, and PMB, to influence the rate of vesicle exchange. Extensive work on PQS and vesicles has shown the ability of PQS to induce curvature in membranes through a wedging mechanism, which increased vesicle production (11, 71). The other compounds are classified as antibiotics, which is not surprising given that the mode of action for a large number of antibiotics is to compromise the bacterial membrane. At high concentrations, these compounds coat the cell membrane, eventually forming pores that lead to cell death (72). At low, subinhibitory concentrations, these compounds may have secondary functions, including the regulation of vesicle exchange. Pore formation does not occur at low concentrations of these molecules (73, 74); instead, binding of these compounds leads to membrane bending and bleb formation, processes known to facilitate vesicle formation (56). Colistin and PMB were previously shown to induce EV formation, although the previous study focused on the ability of EVs to protect bacteria from membrane-targeting antibiotic compounds and phage infection (35). This is not the first time that secondary functions have been identified for antibiotic compounds at sublethal concentrations (74). Low concentrations of fluoroquinolones increased conjugation (75), and subinhibitory concentrations of many antibiotics also act as signaling molecules (73 to 76). Here, we show that regulation of vesicle exchange, and the associated horizontal gene transfer, is yet another potential secondary function of some antibiotic compounds.

There are many such membrane-binding antibiotic compounds, including colistin, nisin, and PMB, and the ability of these compounds to influence vesicle production and uptake does not seem to require a specialized interaction with membrane components. An unknown component found in the supernatants of *E. coli* and *K. pneumoniae* have been shown to increase EV production in *P. aeruginosa* (5). Subinhibitory concentrations of gentamicin also destabilize the membrane and induce vesicle formation in *P. aeruginosa* (77). As shown here, even the eukaryotic compound AS that is involved in membrane restructuring in neurons has the ability to influence vesicle exchange in bacteria. Therefore, many if not all membrane-binding compounds, including other amphipathic alpha-helices, may influence vesicle exchange. Membrane-binding peptides such as antimicrobial peptides are produced by many species of bacteria, suggesting many bacteria may employ these molecules for probable regulation of vesicle exchange, assigning additional function to complex and energy-expensive molecules.

Recently, several mechanisms of vesicle production have been reported (16, 78). Blebbing is one such mechanism, and the compounds tested here may act through this

pathway given their ability to induce curvature in bacterial membranes (14, 35). Others have recently speculated that vesicles loaded by DNA are likely the result of cell explosion (16). It is possible that low concentration of membrane-binding antibiotics contributes to vesicle formation through cell lysis, although it seems unlikely cell lysis accounts for the increased rate of vesicle uptake. Although many recent studies have focused on vesicle production, little work has been done on vesicle uptake by bacteria. The uptake process is essential for the transfer of biomolecules in vesicles, such as genetic material, membrane proteins, regulatory RNAs, and molecules that mediate host–bacterial interactions such as lipopolysaccharides (LPS) (66, 79). Here, we showed that compounds known to restructure the membrane facilitated vesicle uptake, but other mechanisms might also regulate the vesicle uptake rate. In eukaryotic membranes, protein–protein attachments, such as SNARE proteins, are a first step in endocytosis (29). Some proteins on vesicle surfaces even insert into the membrane of recipient cells (80). Recent work suggests that uptake of bacterial vesicles into eukaryotic host cells appears to be rapid (63), although early studies on vesicle uptake via bacteria suggest uptake is a rare event (81). A better understanding of vesicle uptake and the strategies that bacteria have evolved to increase the rate or specificity of vesicle uptake, in addition to the release of membrane structuring molecules, would lead to a better understanding of vesicle exchange and its regulation within bacterial populations.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* lab strain MG1655 was used for all extracellular vesicle and transfer experiments. DH5α was also used in coculture experiments. Bacteria were grown in Luria-Bertani (LB) broth (Difco, Sparks, MD) at 37°C with shaking at 200 rpm. Plasmids were introduced to donor strains via electroporation. Plasmids were maintained in liquid culture with the appropriate antibiotics (VWR, Radnor, PA). A list of plasmids are in Table S1.

Isolation and purification of EVs. EVs were isolated from liquid cultures of *E. coli* MG1655 as previously described (10) with some modifications. Four hundred microliters of overnight culture was used to inoculate 400 mL of LB broth containing selective antibiotic and added exogenous molecule concentration when stated. 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 remaining cells and cellular debris. Vesicles were collected by ultracentrifugation at 80,000 $\times$ *g* (Ti 45 rotor; Beckman Instruments, Inc., Fullerton, CA) at 4°C for 1.5 to 2 h followed by 180,000 $\times$ *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 mL^{-1} of DNase I at 37°C for 20 min followed by deactivation of the DNase I at 80°C for 10 min. Vesicle preparations were also plated on LB agar to check for the presence of bacterial cells.

EV quantification. Extracellular 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 of OmpC/F were determined using ImageJ from a standard curve generated by a BSA protein concentration gradient measured for each experiment. An example standard curve is shown in Fig. S1. Protein concentrations of OmpC/F were used to quantify vesicle concentration and production relative to untreated cells.

Exogenous molecules used. Colistin sulfate salt, nisin, and polymyxin B sulfate were dissolved in deionized water for the preparation of stock solutions, while 2-Heptyl-3-hydroxy-4(1H)-quinolone (PQS) (Sigma-Aldrich Corp., St. Louis, MO) was dissolved in 100% dimethyl sulfoxide (DMSO). As shown in Fig. 4B, this concentration of DMSO did not significantly change the gene transfer time in coculture. This concentration of DMSO, 0.1% vol/vol, was previously not shown to modulate membrane structure (82). Purified alpha-synuclein was provided by Ralf Langen's lab at the University of Southern California (31).

Measurement of bacterial growth. An overnight culture of *E. coli* MG1655 was used to inoculate parallel cultures treated with 1 μg/mL colistin, 10 μg/mL nisin, 1 μg/mL PMB, or 20 μg/mL PQS, at 1% inoculum. The growth of all cultures was monitored in 96 wells at 600 nm using a plate reader (TECAN, infinite M200PRO) for 12 h at 37°C with intermittent shaking for 30 secs.

Propidium iodide assay. Twenty-five milliliters of secondary cultures of *E. coli* MG1655 were grown at 37°C, 200 rpm until optical density (OD) reached ~0.2, after which individual cultures were subjected to the treatment with 1 μg/mL colistin, 10 μg/mL nisin, 1 μg/mL PMB, or 20 μg/mL PQS. Treated cells were harvested at 0, 2, 5, and 10 hours, washed thrice with 1$\times$ PBS, and stained with Propidium Iodide Ready Flow Reagent (Invitrogen by Thermo Fisher Scientific) at 25°C. The culture was again washed with 1$\times$ PBS and fixed with 4% paraformaldehyde (PFA). Five microliters of aliquot from these cultures was then spread on agar pad and imaged with 40$\times$/0.6 NA objective on an ECHO revolve microscope in phase contrast and red fluorescent protein (RFP) channel.

EV-mediated gene transfer. Gene transfer experiments were modified from previously published work (10). The *E. coli* recipient strain was diluted 1:1,000 from overnight culture in 4 mL LB broth (Difco, Sparks, MD) at 37°C with shaking at 200 rpm to early log phase, OD_{600} 0.2, ~2 h, and exogenous molecules were added and incubated for 30 min. Then at time zero hour, purified vesicles were added. The number of vesicles added to recipient cultures was standardized for transfer experiments. In all transfer assays, vesicles equivalent to 1 μg of the outer membrane proteins OmpC/F were used. Every hour, 200 μL of culture was removed and plated on LB agar plates containing either 50 μg mL^{-1} kanamycin, 50 μg mL^{-1} carbenicillin, or both, depending on plasmid resistance. After 16 h of incubation at 37°C, plates were analyzed for the presence of colonies. 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 using PCR. Gain of resistance not associated with plasmid transfer was not observed for any condition tested, and as shown in Fig. 4b, monocultures of each strain did not gain resistance over 12 h of culture.

EV coculture gene transfer. Coculture experiments were performed using DH5α (Δ*lacZ*) cells transformed with pSC101+ (*bla*) and MG1655 (with *lacZ*) transformed with pLC-RK2 (*npr*). Each strain was grown separately starting in overnight cultures and mixed together in 1:1 proportion the next day. The resultant inoculum was added to fresh 100 mL LB at 1% inoculum and grown at 37°C with shaking at 200 rpm for another 12 h. One-milliliter samples withdrawn periodically after every hour from the coculture were washed thrice with 1× PBS to remove compounds in the supernatant, such as secreted beta-lactamase. Washed cells were inoculated in 10 mL LB containing ampicillin (100 μg/mL) and kanamycin (50 μg/mL). Optical densities of these cultures were recorded for all 13 time points (SPECTRONIC 200, Thermo Fisher Scientific) and denoted as OD1. This was followed by incubation of cultures at 37°C and 200 rpm approximately for 12 h and measured for changes in respective optical densities (OD2). The ratio of OD2 to OD1 was used to determine if growth occurred in the presence of selection for both resistance markers. Cultures were streaked on LB agar containing ampicillin and kanamycin to confirm the presence of double transformants. Colonies thus obtained were randomly selected for colony PCR to confirm the presence of *bla* and *npt* genes in transformed cells. (Fig. S10). In addition, cultures at time points with a ratio above 1 were streaked on MacConkey's agar (Sigma-Aldrich) containing ampicillin and kanamycin to differentiate between the two hosts. At this step, an isolated colony was selected to isolate extrachromosomal content and subject it to restriction digestion to further confirm the acquisition of plasmids of interest by the host strain.

Nanoparticle tracking analysis (NTA). Malvern Panalytical Nanosight NS300 was used (Malvern, UK), equipped with a 532-nm green laser. All samples were diluted in PBS to a final volume of 1 mL. For each measurement, five 1-min videos were captured with a detection threshold of 5, embedded laser, and 45 mW. After capture, videos were analyzed by the in-build Nanosight Software NTA 3.1 Build 3.1.46.

Colony PCR. PCR was performed using colonies from transfer assays using One*Taq* (New England BioLabs, Inc., Ipswich, MA). Briefly, the reaction mixtures consisted of 0.5 μL of bacterial colony resuspended in H_2O, 0.2-μM primers, and 1 U of One*Taq* polymerase (New England BioLabs, Inc., Ipswich, MA) in a final volume of 25 μL. The program consisted of 25 cycles of denaturing at 94°C for 30 s, annealing at 60°C for 60 s, and extension at 68°C for 30 s. Primers used for pLC-RK2 were as follows: forward-5′-CATTCGTGATTGCGCCTGAG-3′; reverse-5′-TCAACGGGAAACGTCTTGCT-3′; for pSC101, forward-5′-AGTGATAACACTGCGGCCAA-3′; reverse-5′-TGAGGCACCTATCTCAGCGA-3′.

Restriction digestion. Following gene transfer within the coculture, a randomly selected colony from McConkey's agar containing ampicillin (100 μg/mL) and kanamycin (50 μg/mL) was grown in fresh LB with ampicillin and kanamycin selection. Plasmids were isolated from the culture using QIAprep Spin Miniprep kit, and the isolated plasmids were digested with EcoNI at 37°C for 3 h. Resulting digests were then run on 0.8% agarose gel and compared to samples of controls of digested pLC291, pSC101+, and 1:1 mixtures of pLC291 and pSC101+.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.3 MB.

ACKNOWLEDGMENTS

We thank Ralf Langen's lab for providing the purified sample of alpha-synuclein and for help with transmission electron microscopy, Paolo Neviani and the ExtraCellular Vesicle Core at Children's Hospital Los Angeles for help with nanoparticle analysis, and Carla Vidal Silva for helpful discussion and preliminary work on this project. J.Q.B. and F.T. acknowledge funding from NSF award MCB-1818341, NSF award PHY-1753268, and Army Research Office MURI Award W911NF1910269.

REFERENCES

1. Kim J-Y, Doody AM, Chen DJ, Cremona GH, Shuler ML, Putnam D, DeLisa MP. 2008. Engineered bacterial outer membrane vesicles with enhanced functionality. J Mol Biol 380:51–66. https://doi.org/10.1016/j.jmb.2008.03.076.
2. Berleman J, Auer M. 2013. The role of bacterial outer membrane vesicles for intra- and interspecies delivery. Environ Microbiol 15:347–354. https://doi.org/10.1111/1462-2920.12048.

3. Bonnington K, Kuehn M. 2014. Protein selection and export via outer membrane vesicles. Biochim Biophys Acta 1843:1612–1619. https://doi.org/10.1016/j.bbamcr.2013.12.011.
4. Pathirana RD, Kaparakis-Liaskos M. 2016. Bacterial membrane vesicles: biogenesis, immune regulation and pathogenesis. Cell Microbiol 18:1518–1524. https://doi.org/10.1111/cmi.12658.
5. Horspool AM, Schertzer JW. 2018. Reciprocal cross-species induction of outer membrane vesicle biogenesis via secreted factors. Sci Rep 8:9873. https://doi.org/10.1038/s41598-018-28042-4.
6. Kuehn MJ, Kesty NC. 2005. Bacterial outer membrane vesicles and the host–pathogen interaction. Genes Dev 19:2645–2655. https://doi.org/10.1101/gad.1299905.
7. 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. https://doi.org/10.1128/AEM.66.10.4414-4420.2000.
8. Rumbo C, Fernández-Moreira E, Merino M, Poza M, Mendez JA, Soares NC, Mosquera A, Chaves F, Bou G. 2011. Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: a new mechanism of dissemination of carbapenem resistance genes in *Acinetobacter baumannii*. Antimicrob Agents Chemother 55:3084–3090. https://doi.org/10.1128/AAC.00929-10.
9. 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.
10. Tran F, Boedicker JQ. 2017. Genetic cargo and bacterial species set the rate of vesicle-mediated horizontal gene transfer. Sci Rep 7:9873. https://doi.org/10.1038/s41598-017-07447-7.
11. Schertzer JW, Whiteley M. 2012. A bilayer-couple model of bacterial outer membrane vesicle biogenesis. mBio 3:e00297-11. https://doi.org/10.1128/mBio.00297-11.
12. Pérez-Cruz C, Carrión O, Delgado L, Martinez G, López-Iglesias C, Mercade E. 2013. New type of outer membrane vesicle produced by the Gram-negative bacterium *Shewanella vesiculosa* M7T: implications for DNA content. Appl Environ Microbiol 79:1874–1881. https://doi.org/10.1128/AEM.03657-12.
13. Schwechheimer C, Kulp A, Kuehn MJ. 2014. Modulation of bacterial outer membrane vesicle production by envelope structure and content. BMC Microbiol 14:324. https://doi.org/10.1186/s12866-014-0324-1.
14. MacDonald IA, Kuehn MJ. 2013. Stress-induced outer membrane vesicle production by *Pseudomonas aeruginosa*. J Bacteriol 195:2971–2981. https://doi.org/10.1128/JB.02267-12.
15. Devos S, Van Putte W, Vitse J, Van Driessche G, Stremersch S, Van Den Broek W, Raemdonck K, Braeckmans K, Stahlberg H, Kudryashev M, Savvides SN, Devreese B. 2017. Membrane vesicle secretion and prophage induction in multidrug-resistant *Stenotrophomonas maltophilia* in response to ciprofloxacin stress. Environ Microbiol 19:3930–3937. https://doi.org/10.1111/1462-2920.13793.
16. Turnbull L, Toyofuku M, Hynen AL, Kurosawa M, Pessi G, Petty NK, Osvath SR, Cárcamo-Oyarce G, Gloag ES, Shimoni R, Omasits U, Ito S, Yap X, Monahan LG, Cavaliere R, Ahrens CH, Charles IG, Nomura N, Eberl L, Whitchurch CB. 2016. Explosive cell lysis as a mechanism for the biogenesis of bacterial membrane vesicles and biofilms. Nat Commun 7:11220. https://doi.org/10.1038/ncomms11220.
17. Bernadac A, Gavioli M, Lazzaroni JC, Raina S, Lloubès R. 1998. *Escherichia coli tol-pal* mutants form outer membrane vesicles. J Bacteriol 180:4872–4878. https://doi.org/10.1128/JB.180.18.4872-4878.1998.
18. Roier S, Zingl FG, Cakar F, Durakovic S, Kohl P, Eichmann TO, Klug L, Gadermaier B, Weinzerl K, Prassl R, Lass A, Daum G, Reidl J, Feldman MF, Schild S. 2016. A novel mechanism for the biogenesis of outer membrane vesicles in Gram-negative bacteria. Nat Commun 7:10515. https://doi.org/10.1038/ncomms10515.
19. Schwechheimer C, Kuehn MJ. 2015. Outer-membrane vesicles from Gram-negative bacteria: biogenesis and functions. Nat Rev Microbiol 13:605–619. https://doi.org/10.1038/nrmicro3525.
20. 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.
21. Kim JH, Lee J, Park J, Gho YS. 2015. Gram-negative and Gram-positive bacterial extracellular vesicles. Semin Cell Dev Biol 40:97–104. https://doi.org/10.1016/j.semcdb.2015.02.006.
22. Jackman SL, Regehr WG. 2017. The mechanisms and functions of synaptic facilitation. Neuron 94:447–464. https://doi.org/10.1016/j.neuron.2017.02.047.
23. Walter AM, Böhme MA, Sigrist SJ. 2018. Vesicle release site organization at synaptic active zones. Neurosci Res 127:3–13. https://doi.org/10.1016/j.neures.2017.12.006.
24. Robbins PD, Dorronsoro A, Booker CN. 2016. Regulation of chronic inflammatory and immune processes by extracellular vesicles. J Clin Invest 126:1173–1180. https://doi.org/10.1172/JCI81131.
25. Gai C, Carpanetto A, Deregibus MC, Camussi G. 2016. Extracellular vesicle-mediated modulation of angiogenesis. Histol Histopathol 31:379–391.
26. Todorova D, Simoncini S, Lacroix R, Sabatier F, Dignat-George F. 2017. Extracellular vesicles in angiogenesis. Circ Res 120:1658–1673. https://doi.org/10.1161/CIRCRESAHA.117.309681.
27. Prada I, Meldolesi J. 2016. Binding and fusion of extracellular vesicles to the plasma membrane of their cell targets. Int J Mol Sci 17:1296. https://doi.org/10.3390/ijms17081296.
28. Peñalva DA, Antollini SS, Ambroggio EE, Aveldaño MI, Fanani ML. 2018. Membrane restructuring events during the enzymatic generation of ceramides with very long-chain polyunsaturated fatty acids. Langmuir 34:4398–4407. https://doi.org/10.1021/acs.langmuir.7b04374.
29. Adnan M, Islam W, Zhang J, Zheng W, Lu G-D. 2019. Diverse role of SNARE protein Sec22 in vesicle trafficking, membrane fusion, and autophagy. Cells 8:337. https://doi.org/10.3390/cells8040337.
30. Drin G, Antonny B. 2010. Amphipathic helices and membrane curvature. FEBS Lett 584:1840–1847. https://doi.org/10.1016/j.febslet.2009.10.022.
31. Kegulian NC, Sankhagowit S, Apostolidou M, Jayasinghe SA, Malmstadt N, Butler PC, Langen R. 2015. Membrane curvature-sensing and curvature-inducing activity of islet amyloid polypeptide and its implications for membrane disruption. J Biol Chem 290:25782–25793. https://doi.org/10.1074/jbc.M115.659797.
32. Varkey J, Isas JM, Mizuno N, Jensen MB, Bhatia VK, Jao CC, Petrlova J, Voss JC, Stamou DG, Steven AC, Langen R. 2010. Membrane curvature induction and tubulation are common features of synucleins and apolipoproteins. J Biol Chem 285:32486–32493. https://doi.org/10.1074/jbc.M110.139576.
33. Florez C, Raab JE, Cooke AC, Schertzer JW. 2017. Membrane distribution of the *Pseudomonas* quinolone signal modulates outer membrane vesicle production in *Pseudomonas aeruginosa*. mBio 8:e01034-17. https://doi.org/10.1128/mBio.01034-17.
34. Schlatterer K, Beck C, Hanzelmann D, Lebtig M, Fehrenbacher B, Schaller M, Ebner P, Nega M, Otto M, Kretschmer D, Peschel A. 2018. The mechanism behind bacterial lipoprotein release: phenol-soluble modulins mediate Toll-like receptor 2 activation via extracellular vesicle release from *Staphylococcus aureus*. mBio 9:e01851-18. https://doi.org/10.1128/mBio.01851-18.
35. Manning AJ, Kuehn MJ. 2011. Contribution of bacterial outer membrane vesicles to innate bacterial defense. BMC Microbiol 11:258. https://doi.org/10.1186/1471-2180-11-258.
36. Dupuy FG, Pagano I, Andenoro K, Peralta MF, Elhady Y, Heinrich F, Tristram-Nagle S. 2018. Selective interaction of colistin with lipid model membranes. Biophys J 114:919–928. https://doi.org/10.1016/j.bpj.2017.12.027.
37. D'Souza-Schorey C, Schorey JS. 2018. Regulation and mechanisms of extracellular vesicle biogenesis and secretion. Essays Biochem 62:125–133. https://doi.org/10.1042/EBC20170078.
38. Sedgwick AE, D'Souza-Schorey C. 2018. The biology of extracellular microvesicles. Traffic 19:319–327. https://doi.org/10.1111/tra.12558.
39. French KC, Antonyak MA, Cerione RA. 2017. Extracellular vesicle docking at the cellular port: extracellular vesicle binding and uptake. Semin Cell Dev Biol 67:48–55. https://doi.org/10.1016/j.semcdb.2017.01.002.
40. Ottolini D, Cali T, Szabò I, Brini M. 2017. Alpha-synuclein at the intracellular and the extracellular side: functional and dysfunctional implications. Biol Chem 398:77–100. https://doi.org/10.1515/hsz-2016-0201.
41. Plotegher N, Berti G, Ferrari E, Tessari I, Zanetti M, Lunelli L, Greggio E, Bisaglia M, Veronesi M, Girotto S, Dalla Serra M, Perego C, Casella L, Bubacco L. 2017. DOPAL derived alpha-synuclein oligomers impair synaptic vesicles physiological function. Sci Rep 7:40699. https://doi.org/10.1038/srep40699.
42. Masaracchia C, Hnida M, Gerhardt E, Lopes da Fonseca T, Villar-Pique A, Branco T, Stahlberg MA, Dean C, Fernández CO, Milosevic I, Outeiro TF. 2018. Membrane binding, internalization, and sorting of alpha-synuclein in the cell. Acta Neuropathol Commun 6:79. https://doi.org/10.1186/s40478-018-0578-1.
43. Pranke IM, Morello V, Bigay J, Gibson K, Verbavatz J-M, Antonny B, Jackson CL. 2011. α-Synuclein and ALPS motifs are membrane curvature sensors whose contrasting chemistry mediates selective vesicle binding. J Cell Biol 194:89–103. https://doi.org/10.1083/jcb.201011118.
44. Park S-C, Moon JC, Shin SY, Son H, Jung YJ, Kim N-H, Kim Y-M, Jang M-K, Lee JR. 2016. Functional characterization of alpha-synuclein protein with

antimicrobial activity. Biochem Biophys Res Commun 478:924–928. https://doi.org/10.1016/j.bbrc.2016.08.052.
45. Warren HS, Kania SA, Siber G. 1985. Binding and neutralization of bacterial lipopolysaccharide by colistin nonapeptide. Antimicrob Agents Chemother 28:107–112. https://doi.org/10.1128/AAC.28.1.107.
46. Wiedemann I, Breukink E, van Kraaij C, Kuipers OP, Bierbaum G, de Kruijff B, Sahl H-G. 2001. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J Biol Chem 276:1772–1779. https://doi.org/10.1074/jbc.M006770200.
47. Santos DE, Pol-Fachin L, Lins RD, Soares TA. 2017. Polymyxin binding to the bacterial outer membrane reveals cation displacement and increasing membrane curvature in susceptible but not in resistant lipopolysaccharide chemotypes. J Chem Inf Model 57:2181–2193. https://doi.org/10.1021/acs.jcim.7b00271.
48. Lin J, Cheng J, Wang Y, Shen X. 2018. The *Pseudomonas* quinolone signal (PQS): not just for quorum sensing anymore. Front Cell Infect Microbiol 8:230. https://doi.org/10.3389/fcimb.2018.00230.
49. Cannatelli A, Giani T, Aiezza N, Di Pilato V, Principe L, Luzzaro F, Galeotti CL, Rossolini GM. 2017. An allelic variant of the PmrB sensor kinase responsible for colistin resistance in an *Escherichia coli* strain of clinical origin. Sci Rep 7:1–6. https://doi.org/10.1038/s41598-017-05167-6.
50. Matsumoto Y, Hayama K, Sakakihara S, Nishino K, Noji H, Iino R, Yamaguchi A. 2011. Evaluation of multidrug efflux pump inhibitors by a new method using microfluidic channels. PLoS One 6:e18547. https://doi.org/10.1371/journal.pone.0018547.
51. Kwak YG, Jacoby GA, Hooper DC. 2013. Induction of plasmid-carried *qnrS1* in *Escherichia coli* by naturally occurring quinolones and quorum-sensing signal molecules. Antimicrob Agents Chemother 57:4031–4034. https://doi.org/10.1128/AAC.00337-13.
52. Hoshiko Y, Nishiyama Y, Moriya T, Kadokami K, Lopez Jacome LE, Hirano R, García-Contreras R, Maeda T. 2021. Quinolone signals related to *Pseudomonas* quinolone signal-quorum sensing inhibits the predatory activity of *Bdellovibrio bacteriovorus*. Front Microbiol 12:2549. https://doi.org/10.3389/fmicb.2021.722579.
53. Toyofuku M, Nakajima-Kambe T, Uchiyama H, Nomura N. 2010. The effect of a cell-to-cell communication molecule, *Pseudomonas* quinolone signal (PQS), produced by *P. aeruginosa* on other bacterial species. Microbes Environ 25:1–7. 0912140139-0912140139. https://doi.org/10.1264/jsme2.ME09156.
54. Mashburn LM, Whiteley M. 2005. Membrane vesicles traffic signals and facilitate group activities in a prokaryote. Nature 437:422–425. https://doi.org/10.1038/nature03925.
55. Bitto NJ, Zavan L, Johnston EL, Stinear TP, Hill AF, Kaparakis-Liaskos M. 2021. Considerations for the analysis of bacterial membrane vesicles: methods of vesicle production and quantification can influence biological and experimental outcomes. Microbiol Spectr 9:e01273-21. https://doi.org/10.1128/Spectrum.01273-21.
56. MacDonald IA, Kuehn MJ. 2012. Offense and defense: microbial membrane vesicles play both ways. Res Microbiol 163:607–618. https://doi.org/10.1016/j.resmic.2012.10.020.
57. Kuzmin PI, Zimmerberg J, Chizmadzhev YA, Cohen FS. 2001. A quantitative model for membrane fusion based on low-energy intermediates. Proc Natl Acad Sci U S A 98:7235–7240. https://doi.org/10.1073/pnas.121191898.
58. Schmidt NW, Wong GC. 2013. Antimicrobial peptides and induced membrane curvature: geometry, coordination chemistry, and molecular engineering. Curr Opin Solid State Mater Sci 17:151–163. https://doi.org/10.1016/j.cossms.2013.09.004.
59. Schwechheimer C, Sullivan CJ, Kuehn MJ. 2013. Envelope control of outer membrane vesicle production in Gram-negative bacteria. Biochemistry 52:3031–3040. https://doi.org/10.1021/bi400164t.
60. Mell JC, Redfield RJ. 2014. Natural competence and the evolution of DNA uptake specificity. J Bacteriol 196:1471–1483. https://doi.org/10.1128/JB.01293-13.
61. 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.
62. Cabezón E, Ripoll-Rozada J, Peña A, De La Cruz F, Arechaga I. 2015. Towards an integrated model of bacterial conjugation. FEMS Microbiol Rev 39:81–95. https://doi.org/10.1111/1574-6976.12085.
63. 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.
64. Boto L. 2010. Horizontal gene transfer in evolution: facts and challenges. Proc Biol Sci 277:819–827. https://doi.org/10.1098/rspb.2009.1679.
65. Kloos D-U, Strätz M, Güttler A, Steffan RJ, Timmis KN. 1994. Inducible cell lysis system for the study of natural transformation and environmental fate of DNA released by cell death. J Bacteriol 176:7352–7361. https://doi.org/10.1128/jb.176.23.7352-7361.1994.
66. Bonnington KE, Kuehn MJ. 2016. Outer membrane vesicle production facilitates LPS remodeling and outer membrane maintenance in *Salmonella* during environmental transitions. mBio 7:e01532-16. https://doi.org/10.1128/mBio.01532-16.
67. Bos J, Cisneros LH, Mazel D. 2021. Real-time tracking of bacterial membrane vesicles reveals enhanced membrane traffic upon antibiotic exposure. Sci Adv 7:eabd1033. https://doi.org/10.1126/sciadv.abd1033.
68. Kulp A, Kuehn MJ. 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annu Rev Microbiol 64:163–184. https://doi.org/10.1146/annurev.micro.091208.073413.
69. Elhenawy W, Bording-Jorgensen M, Valguarnera E, Haurat MF, Wine E, Feldman MF. 2016. LPS remodeling triggers formation of outer membrane vesicles in *Salmonella*. mBio 7:e00940-16. https://doi.org/10.1128/mBio.00940-16.
70. Mondal Roy S, Sarkar M. 2011. Membrane fusion induced by small molecules and ions. J Lipids 2011:528784. https://doi.org/10.1155/2011/528784.
71. Bohuszewicz O, Liu J, Low HH. 2016. Membrane remodelling in bacteria. J Struct Biol 196:3–14. https://doi.org/10.1016/j.jsb.2016.05.010.
72. Brogden KA. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3:238–250. https://doi.org/10.1038/nrmicro1098.
73. Linares JF, Gustafsson I, Baquero F, Martinez J. 2006. Antibiotics as intermicrobial signaling agents instead of weapons. Proc Natl Acad Sci U S A 103:19484–19489. https://doi.org/10.1073/pnas.0608949103.
74. Andersson DI, Hughes D. 2014. Microbiological effects of sublethal levels of antibiotics. Nat Rev Microbiol 12:465–478. https://doi.org/10.1038/nrmicro3270.
75. Shun-Mei E, Zeng J-M, Yuan H, Lu Y, Cai R-X, Chen C. 2018. Sub-inhibitory concentrations of fluoroquinolones increase conjugation frequency. Microb Pathog 114:57–62. https://doi.org/10.1016/j.micpath.2017.11.036.
76. Wang Y, Kern SE, Newman DK. 2010. Endogenous phenazine antibiotics promote anaerobic survival of *Pseudomonas aeruginosa* via extracellular electron transfer. J Bacteriol 192:365–369. https://doi.org/10.1128/JB.01188-09.
77. Kadurugamuwa J, Beveridge T. 1997. Natural release of virulence factors in membrane vesicles by *Pseudomonas aeruginosa* and the effect of aminoglycoside antibiotics on their release. J Antimicrob Chemother 40:615–621. https://doi.org/10.1093/jac/40.5.615.
78. Cooke AC, Nello AV, Ernst RK, Schertzer JW. 2019. Analysis of *Pseudomonas aeruginosa* biofilm membrane vesicles supports multiple mechanisms of biogenesis. PLoS One 14:e0212275. https://doi.org/10.1371/journal.pone.0212275.
79. Nevot M, Deroncelé V, Messner P, Guinea J, Mercadé E. 2006. Characterization of outer membrane vesicles released by the psychrotolerant bacterium *Pseudoalteromonas antarctica* NF3. Environ Microbiol 8:1523–1533. https://doi.org/10.1111/j.1462-2920.2006.01043.x.
80. Mulcahy LA, Pink RC, Carter DRF. 2014. Routes and mechanisms of extracellular vesicle uptake. J Extracellular Vesicles 3:24641. https://doi.org/10.3402/jev.v3.24641.
81. Nazarian P, Tran F, Boedicker JQ. 2018. Modeling multispecies gene flow dynamics reveals the unique roles of different horizontal gene transfer mechanisms. Front Microbiol 9:2978. https://doi.org/10.3389/fmicb.2018.02978.
82. Dyrda G, Boniewska-Bernacka E, Man D, Barchiewicz K, Słota R. 2019. The effect of organic solvents on selected microorganisms and model liposome membrane. Mol Biol Rep 46:3225–3232. https://doi.org/10.1007/s11033-019-04782-y.