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DOI:
10.1016/j.mib.2020.02.002

Document Version
Peer reviewed version

Citation for published version (Harvard):
McInnes, RS, McCallum, GE, Lamberte, LE & van Schaik, W 2020, 'Horizontal transfer of antibiotic resistance genes in the human gut microbiome', *Current Opinion in Microbiology*, vol. 53, pp. 35-43. https://doi.org/10.1016/j.mib.2020.02.002

Horizontal transfer of antibiotic resistance genes in the human gut microbiome

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Abstract

Infections caused by antibiotic-resistant bacteria are a major threat to public health. The pathogens causing these infections can acquire antibiotic resistance genes in a process termed horizontal gene transfer (HGT). HGT is a common event in the human gut microbiome, i.e. the microbial ecosystem of the human intestinal tract. HGT in the gut microbiome can occur via different mechanisms of which transduction and conjugation have been best characterised. Novel bioinformatic tools and experimental approaches have been developed to determine the association of antibiotic resistance genes with their microbial hosts and to quantify the extent of HGT in the gut microbiome. Insights from studies into HGT in the gut microbiome may lead to the development of novel interventions to minimise the spread of antibiotic resistance genes among commensals and opportunistic pathogens.

Highlights

- The human gut microbiome harbours antibiotic resistance genes ('the resistome')
- Antibiotic resistance genes can spread in the gut microbiome through horizontal gene transfer (HGT)
- The best characterised mechanisms of HGT in the human gut microbiome are transduction and conjugation
- Novel bioinformatic and experimental approaches have been developed to study HGT in the gut

The human gut microbiome and antimicrobial resistance

The term ‘human gut microbiome’ describes the microorganisms, their genomes and the environmental conditions of the human intestinal tract [1]. Over the last decade, the implementation of high-throughput, low-cost sequencing methods has fuelled research into the gut microbiome, with the aim to uncover its composition, function, and role in health and disease [2]. The gut contains hundreds of bacterial species, collectively called the microbiota, with those belonging to the phyla Bacteroidetes and Firmicutes accounting for 90% of all species in the gut of healthy adults [3]. The phyla Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia are generally less abundant, but can contribute to important functions, such as providing protection against invading pathogens [4,5]. Most gut bacteria have a symbiotic or commensal relationship with the human host. However, the gut microbiota also contains opportunistic pathogens, including those belonging to the families Enterobacteriaceae, particularly *Escherichia coli* and *Klebsiella pneumoniae*, and Enterococcaceae, most notably *Enterococcus faecalis* and *Enterococcus faecium*. Intestinal carriage of opportunistic pathogens can predispose an individual to urinary tract infections and, in immunocompromised patients, more serious systemic infections [6–8]. Recent decades have seen a global rise of infections caused by antibiotic-resistant clones of *E. coli*, *K. pneumoniae* [9], and *E. faecium* [10].

Infections caused by antibiotic-resistant bacteria are a significant threat to global public health. The annual attributable mortality due to antibiotic-resistant infections in the EU alone is estimated to number over 30,000 deaths, with the highest cases occurring in Italy and Greece [11]. Morbidity and mortality due to multi-drug resistant

infections are even higher in low- and middle-income countries in Asia, Africa and South America [12–14].

The main mechanisms by which bacteria can become resistant to antibiotics are prevention of the antibiotic from reaching toxic levels inside the cell, modification of the antibiotic target, and modification or degradation of the antibiotic itself [15]. These resistance mechanisms can arise through mutations of chromosomal genes and the acquisition of antibiotic resistance genes (ARGs) from other strains of the same, or different, species in a process termed horizontal gene transfer (HGT). The sharing of genes through HGT contributes importantly to the global dissemination of ARGs [16]. HGT can occur in any environment, particularly when bacterial loads are high, e.g. in soil [17], in wastewater treatment plants [18,19] and in the gut microbiome of humans and animals [20–22].

As *E. coli*, *K. pneumoniae*, *C. difficile,* enterococci and other opportunistic pathogens inhabit the human gut, there is a distinct possibility that they can acquire resistance determinants from other members of the gut microbiota. Indeed, previous work has shown that the gut harbours many and diverse antibiotic resistance determinants, which have collectively been termed the ‘gut resistome’ [23,24]. In this review, we will provide an overview of the different mechanisms of HGT in the human gut microbiome and of innovative methods to study HGT in this microbial ecosystem.

Mechanisms of horizontal gene transfer

ARGs can be horizontally transferred through a number of mechanisms, the most important of which are transformation, transduction, and conjugation. More recently,

the role of membrane vesicles (MVs) in HGT has also been recognised. In this section, we will shortly discuss the relevance of these HGT mechanisms in the context of the spread of ARGs in the gut microbiome (Figure 1).

In transformation, naked DNA from the extracellular environment is taken up by bacteria and inserted into their genomes. The process of transformation requires bacteria to be naturally transformable or competent. Over 80 bacterial species have been experimentally proven to be naturally competent, and the presence of genes involved in DNA uptake in a number of other species suggests that this trait is more widespread [25]. The stimuli that lead to a competent state in naturally transformable bacteria are only partially understood, but nutrient starvation and the presence of competence-inducing peptides have been identified as triggers [26]. Important pathogens, including *Neisseria gonorrhoeae*, *Vibrio cholerae*, and *Streptococcus pneumoniae* are naturally competent pathogens and have acquired antibiotic resistance through this process [27]. While DNAse activity in the gut will degrade most DNA [28], intact plasmid DNA can be isolated from the gut contents of rats that are fed plasmids [29]. This finding indicates that there is potential for extracellular DNA to be taken up by naturally competent bacteria. Observations showing that *E. coli* can be transformed by plasmid DNA under natural conditions [30,31], suggest that *E. coli* could take up DNA in the gut, with transformation thus conceivably contributing to the spread of ARGs. The extent by which DNA can spread horizontally in the gut through the process of transformation is, however, currently unknown.

MVs Membrane vesicles (MVs) are 20–250 nm spherical structures that are predominantly produced by Gram-negative bacteria when the outer membrane

bulges away from the cell and is then released through constriction [32]. MVs fuse with their target cells, thus delivering their cargo. MVs that are produced *in vitro* by the gut commensal *Bacteroides* can contain β-lactamases and these vesicles protect target cells against β-lactam antibiotics [33]. In the context of HGT, it is relevant that MVs produced by gut bacteria can also contain cytoplasmic contents, including DNA [34]. DNA-containing MVs are thought to be formed by protrusion of both the outer and inner membrane, which leads to the inclusion of cytoplasmic components into the vesicles [35]. Indeed, membrane vesicles isolated from bacteria in the genus *Acinetobacter* can transfer antibiotic resistance plasmids *in vitro* [36]. Similarly, vesicle-mediated transfer of DNA has also been reported for *E. coli* [37]. While MVs are produced in the gut and can potentially influence host immune responses [38], it is as yet not clear whether they can also contribute to HGT in the gut microbiome.

Transduction describes the transfer of chromosomal and extrachromosomal DNA between bacteria via a viral intermediate known as a bacteriophage. The main mechanisms of transduction are generalised transduction, specialised transduction, and lateral transduction. Collectively these can cause the mobilisation of any fragment of a bacterial genome [39]. Generalised transduction occurs when bacteriophages in the lytic cycle incorporate sections of the DNA of the bacterial host during capsid synthesis. In specialised transduction, regions immediately flanking the integration site of a lysogenic phage are excised and packaged into the capsid [39]. Lateral transduction, in contrast, occurs when prophages initiate DNA replication while they are still integrated into the host. This process generates multiple copies of DNA before excision from the host genome occurs. Once excised, the DNA – which may contain both phage and the adjacent genes up to hundreds of kilobases in length – are packaged into new phage particles and transferred to other bacterial

128 strains [40]. The human gut contains an extensive community of bacteriophages
129 [41,42] and significant numbers of ARG-carrying phages are present in the human
130 gut and other environments [43]. The abundance of these ARG-carrying phages in
131 the human gut increases upon antibiotic treatment [●44]. Experiments in mouse
132 models have shown that transduction is a driving force behind genetic diversity in
133 gut-colonising *E. coli* strains [●45] and can contribute to the emergence of drug
134 resistance in gut bacteria [46]. However, the quantitative contribution of phages to
135 the horizontal transfer of ARGs is still poorly understood. We foresee that novel
136 methods to detect and quantify HGT in the gut (further outlined below) will be
137 implemented to address this question.

138 In conjugation, mobile genetic elements such as plasmids and integrative and
139 conjugative elements (ICEs) are transferred from one bacterium to another [47].
140 Among conjugative elements, conjugative plasmids are arguably the most relevant
141 for the spread of ARGs as they have the potential to carry multiple resistance genes,
142 due to their substantial size (median of 90 kbp) and the common occurrence of one
143 or more toxin-antitoxin modules which ensure that plasmids are retained within their
144 microbial hosts [48]. In addition, conjugative plasmids frequently carry genes, other
145 than ARGs, that contribute to microbial fitness, e.g. by coding for novel metabolic
146 routes or tolerance to disinfectants or heavy metals. Antibiotic resistance
147 determinants can thus be co-selected under a variety of conditions as they share a
148 genetic platform with other fitness determinants [48]. Conjugation is a complex,
149 multistage, and contact-dependent process where DNA is transported via a pilus
150 between bacteria in close proximity to each other [49]. The gut, with its high density
151 of bacterial cells and dense mucus layer, provides an environment conducive to
152 conjugation. The spread of antibiotic resistance plasmids and ICEs has been

observed among commensals and opportunistic pathogens while colonising the human gut [50–52]. Notably, conjugative plasmids can provide the machinery allowing the mobilisation of DNA that is not self-transmissible, thus greatly increasing the potential for HGT of resistance determinants [53].

Bioinformatic tools to study HGT in the gut

The high-throughput sequencing of DNA isolated from microbial ecosystems, often referenced to as shotgun metagenomics, is widely used to study the microbial diversity of the human gut microbiome [54]. The Illumina DNA sequencing platform is most commonly used for shotgun metagenomic studies of the gut microbiome. The relatively short sequence reads generated by Illumina sequencers can be used to detect and quantify the abundance of ARGs in the human gut microbiome [55]. It is, however, difficult to resolve linkage of ARGs and MGEs as these elements are generally rich in DNA repeats that are difficult to resolve with short reads [56]. The advent of long-read technologies such as Oxford Nanopore and PacBio sequencing allows the reconstruction of genome and plasmid sequences from complex metagenomic samples [19]. Independent of the platform used for shotgun metagenomics, approaches based on DNA sequencing have long struggled to identify the microbial hosts of ARGs and to detect HGT events. Recently, advanced bioinformatic tools have been developed to address this issue (Table 1).

Different approaches have been developed to identify HGT events in metagenomic sequencing data [●57]. These tools include MetaCHIP, which uses a combination of best-match and phylogenetic approaches to identify HGT candidates from genes that have a different taxonomic assignment to the contig or genome they have been

assembled into [58]. An additional method, MetaCherchant, has been developed which uses local de Bruijn graph assembly to uncover the wider context of ARGs. When metagenomes from a time series are compared, a change in gene context suggests that an HGT event has occurred [59]. A recently developed binning technique which utilises the methylation patterns of DNA, as determined by Oxford Nanopore and PacBio sequencing, has proven useful in linking MGEs to their host. Methylation motifs are given scores and DNA sequences from MGE and from bacterial genomes can be matched based on their methylation scores thus identifying the host of the MGE [●60]. If the same MGE is identified in multiple hosts then an HGT event may have occurred. However, purely metagenomic methods are still limited in their ability to accurately identify all HGT events, because the conjugative transfer of ARG-carrying plasmids between different microbial hosts cannot be resolved by DNA sequencing alone. For this reason, novel experimental approaches have been developed to characterise HGT in the gut microbiome (Table 1; Figure 2).

Novel experimental approaches to study HGT in the gut

HGT has been studied in the human gut since the 1960s [61]. In these human challenge studies, bacteria capable of transferring DNA (‘donors’’) and recipient strains were administered to the host and transconjugants were then isolated by selective culture from the host faeces. However, these studies are fraught with ethical challenges as there is a risk that multidrug resistant donor strains or transconjugants colonize the gut of the volunteers, and thus animal models are more frequently used to study HGT in the gut (Figure 2A). More recently, culturing of

human faeces on selective media has identified novel hosts of ARGs, such as the *vanB* vancomycin resistance transposon in the gut commensals *Eggerthella lenta* and *Clostridium innocuum* [62], which can be transferred to the opportunistic pathogen *E. faecium* in the mouse gut [63]. These findings support the hypothesis that gut commensals act as a reservoir for ARGs. A drawback of these approaches is that they require the recipient cells to be cultured and while important advances have been made in the isolation of gut commensals [64], it remains technically challenging to systematically identify strains that acquire ARGs and uncover the hidden network of HGT in the gut.

In recent years, novel techniques have been developed which allow the high-throughput characterisation of HGT events in microbial communities. The inclusion of fluorescent markers on plasmids has enabled the use of technologies such as confocal fluorescence microscopy and Fluorescence Activated Cell Sorting (FACS) to uncover the host range of plasmids (Figure 2B). In one study, HGT events were identified by the use of plasmids that carry a green fluorescent protein gene (*gfp*) of which expression was controlled by a chromosomally encoded repressor. Upon conjugation of the plasmid to a new host, repression of *gfp* was lifted and transconjugants could be identified by their green fluorescence [65]. Transconjugants were then isolated using FACS and subsequently subjected to 16S rRNA gene sequencing to identify which hosts the plasmids had spread to. A drawback of this approach is that typically only one plasmid is examined at a time, which limits our understanding of the wider picture of HGT events within a microbiome. In addition, there is a distinct lack of genetic tools to perform these studies in microbial hosts other than model strains like *E. coli* MG1655.

Several techniques have been developed that physically link ARGs to their microbial hosts in microbiomes. These data can be used to elucidate the patterns of ARG dissemination in microbiomes (Figure 2C). In Emulsion, Paired Isolation, and Concatenation PCR (epicPCR), single microbial cells are encapsulated in polyacrylamide beads followed by a fusion PCR step within individual beads. This results in a concatemer of the targeted gene (e.g. an ARG) and the 16S rRNA gene, which serves as a phylogenetic marker [66]. The resulting fusion PCR amplicon can be sequenced using Illumina technology to identify the host(s) of the gene targeted in epicPCR. Recently, epicPCR has been used to determine the diversity of ARG hosts in wastewater [67].

Another technique that can be used to identify the bacterial hosts of ARGs is chromosome conformation capture (3C). During 3C, cells are incubated with formaldehyde to cross-link the DNA, followed by restriction digestion, ligation of the cross-linked DNA and finally reversal of the cross-linking [68]. Sequencing the resulting 3C library will then reveal cross-links between MGEs carrying ARGs and chromosomal DNA, thus allowing the host of the MGEs to be identified. Metagenomic 3C (meta3C) was performed on mouse gut microbiome samples to link phage sequences to their bacterial hosts [●69]. Recently, a derivative of 3C called Hi-C, which includes an additional step to enrich for ligated fragments of DNA, has been used to link ARGs to their bacterial hosts in a variety of complex samples. By performing Hi-C on a wastewater sample, the microbial hosts of ARGs and *in situ* host ranges of plasmids were assessed, where the authors found that the phylum Bacteroidetes was one of the most common reservoirs of ARGs in the sample [70]. Also using Hi-C data, in combination with long-read assembly, the microbial hosts of viruses and ARGs were linked in a cow rumen microbiome [●71]. Hi-C has similarly

been performed on human gut microbiome samples. In a pioneering study, an approximately 600-kbp megaplasmid was linked to several species in the order Clostridiales [72]. More recently, the transfer of accessory elements was tracked in two human stool samples collected 10 years apart using 3C, but the authors did not discuss linkage of ARGs to their microbial hosts [73]. While there has, thus far, been no extensive study using 3C or its derivatives to link ARGs to their host in a human gut microbiome sample, these studies have provided a foundation for the use of these novel techniques to study HGT in the gut.

A recent study [●●74] described an elegant recorder for HGT events based on the ability of the CRISPR-Cas9 system to integrate fragments of invading DNA into the bacterial genome (Figure 2D). CRISPR-Cas9 is an adaptive immune system that bacteria use to defend against invasion by foreign DNA [75]. Invading DNA is incorporated into the bacterial genome at the CRISPR locus by the Cas1-Cas2 complex in a process known as spacer acquisition. These sequences are subsequently transcribed and used to target the Cas9 nuclease to invading DNA which matches the spacer sequence [76]. To record HGT events, Munck and co-workers engineered an *E. coli* strain containing a plasmid with the *cas1-cas2* operon under the control of an inducible promoter to capture DNA sequences which have entered the cell. Sequencing of the CRISPR spacer regions was then used to not only identify HGT events but also the order in which the events occurred. When this system was applied to clinical faecal samples, it showed that HGT is frequent in the gut and that IncX-type plasmids transferred most actively [●●74]. This technique promises to uncover HGT events at an unprecedented resolution, but to unveil the full extent of the gut mobilome it will need to be adapted to function in bacteria which fall outside of the Enterobacteriaceae family.

Conclusions and future perspectives

The human gut microbiome contains a wide variety of ARGs. Modern sequencing-based and experimental technologies are uncovering the extent by which resistant genes spread between strains and species that inhabit the gut. Genes that confer resistance against clinically relevant antibiotics and are carried on mobile genetic elements that replicate in pathogens are considered to be an immediate threat to the successful treatment of clinical infections [77]. While bacteriophages can potentially mobilise any chromosomal gene or mutation, the conjugative spread of plasmids is still widely considered to be the most important way resistance genes can be transferred among bacteria.

The observation that ARGs from opportunistic pathogens can also be found in the genomes of Gram-positive commensal bacteria [62,78] suggests that HGT of ARGs in the gut is pervasive, particularly among the Firmicutes. Carbapenemases and extended-spectrum beta-lactamases that are increasingly prevalent in the opportunistic pathogens *E. coli* and *K. pneumoniae* can readily spread among Proteobacteria in the gut [79,80] but HGT to other phyla have, to our knowledge, not been reported. The scarcity of HGT of resistance genes across phyla can be explained by the specific physiological contexts in which proteins conferring antibiotic resistance need to operate, which makes it unlikely that resistance genes can be functional, at a low biological cost, in phylogenetically disparate bacteria [●●81]. However, soil microcosm experiments have provided evidence that plasmids can spread, albeit infrequently, among diverse phyla [65]. As HGT is a common event in the gut microbiome [●●74], it is not unlikely that broad-host range MGEs carrying

resistance genes will start to disseminate across phyla, although intra-phylum HGT remains quantitatively more important. There is still a lack of knowledge on the conditions in the gut microbiome that favour HGT events, but inflammation of the host and the production of membrane-destabilizing agents by both the microbiota and the host have been proposed to promote HGT in the gut [82].

Our improved understanding of HGT in the gut opens up avenues for novel interventions to minimise transfer of ARGs, e.g. by the conjugative delivery of CRISPR-Cas to selectively deplete strains carrying resistance genes [●83]. We envisage that the novel techniques reviewed here will aid in further elucidating the exchange networks of ARGs in the human gut. The information gleaned from these studies can then support the development of targeted approaches to control or reduce the number of antibiotic-resistant bacteria in the human gut, particularly in individuals that are at high risk of developing infections, such as neonates, the immunocompromised, and the elderly.

Acknowledgments

R.S.M is funded by the Wellcome Trust Antimicrobials and Antimicrobial Resistance Doctoral Training Programme (215154/Z/18/Z). G.E.M. is funded by the MRC Impact Doctoral Training Programme (MR/N013913/1). W.v.S. and L.E.L. are funded through BBSRC (BB/S017941/1). Work in the lab of W.v.S. is also supported by a Royal Society Wolfson Merit Award (WM160092).

Table

	Technique		Description	Limitations	Ref
Bioinformatics	MetaCHIP		Identifies differences in taxonomic assignments between genes and the contig or genome they have been assembled into	Detection of recent HGT events is challenging	[58]
	MetaCherchant		Uses local De Bruijn graph assembly to uncover wider context of ARGs	HGT of high copy-number MGEs may be difficult to detect due to complexity of the assembly graph	[59]
	Binning by DNA methylation		DNA sequences from MGEs and bacterial genomes are matched based on their methylation	High numbers of genomes in sample limit uniqueness of methylation signal	[●60]
Experimental	Culturing approaches	*in vivo* studies	Strains with MGEs are administered to a host and transconjugants are then isolated from faecal samples by culture	Ethical challenges; low throughput	[50]
		culturomics	High-throughput culture-based methods to identify bacterial hosts of antibiotic resistance genes	Laborious and technically challenging, particularly for bacteria that are present at low abundance	[64]
	Fluorescent reporter systems		Fluorescently-tagged plasmids are used to track the movement of plasmids in complex microbiomes	Needs to be optimised for each plasmid-host combination	[65]
	epicPCR		Single microbial cells are captured in polyacrylamide beads, followed by fusion PCR on a target and the 16S rRNA gene, and sequencing of the amplicon	Targets only one gene per experiment; uses 16S rRNA gene as a low-resolution phylogenetic marker; requires multiple rounds of PCR introducing biases	[66]
	Chromosome conformation capture	meta3C	By physically cross-linking DNA inside microbial cells, and subsequent sequencing of the cross-linked DNA, the hosts of genes of interest are identified	Requires high sequencing depth; can be difficult to accurately resolve hosts to strain- or species-level	[●69,73]
		Hi-C	Similar to meta3C but with an additional step to enrich for cross-linked fragments of DNA before sequencing.		[70–72]
	CRISPR recorder		A CRISPR-based system that utilises the spacer acquisition process to record HGT events. Uniquely able to track transient HGT events.	The system has presently only been developed for *E. coli*	[●●74]

Table 1. Summary of current approaches to study horizontal gene transfer in the gut microbiome. Bioinformatic approaches to identify HGT have recently been reviewed in [●57] and the tools highlighted here are a selection of those that have recently been developed. All bioinformatic tools listed in this table for detecting HGT

328 in metagenomes are reliant on accurate and contiguous metagenomic assembly.

329 Ref: references.

330

Figure legends

Figure 1. Mechanisms of horizontal gene transfer (HGT).

Transformation: physiologically competent bacteria can take up naked DNA from the environment. **Membrane vesicle fusion:** 20-250 nm spherical, lipid bilayer-enclosed vesicles can transport cargo between bacteria, including DNA. **Transduction:** genetic material can be transferred between donor and recipient bacteria via a bacteriophage intermediate. **Conjugation:** mobile genetic elements, such as plasmids, can transfer via a pilus formed between donor and recipient cells. The mechanisms of HGT illustrated in this figure can mediate the transfer of both chromosomal and extra-chromosomal DNA.

Figure 2. Experimental approaches to study horizontal gene transfer (HGT) in the gut.

Panel A: the use of culture-based animal models to study HGT. Laboratory animals are fed bacteria containing an antibiotic resistance gene (ARG)-plasmid and stool samples can then be cultured on antibiotic-containing media to isolate transconjugants. **Panel B:** fluorescent reporter systems to identify HGT events. A donor cell carries a green-fluorescent protein (GFP)-tagged plasmid and a repressor gene on the chromosome. GFP will only be expressed when the plasmid is transferred to another cell that does not contain the repressor and GFP-producing transconjugants can thus be isolated using fluorescence-activated cell sorting (FACS). **Panel C:** novel techniques for physically linking ARGs to their microbial

host. Emulsion, Paired Isolation, and Concatenation PCR (epicPCR) involves the encapsulation of single bacterial cells in polyacrylamide beads. After cell lysis, a PCR is performed in each bead individually. The PCR targets an ARG with custom primers where the reverse primer contains an overhang homologous to the 16S rRNA gene, of which another conserved region is targeted by a third primer. The resulting fusion PCR amplicon contains the ARG fused with the 16S rRNA gene of its host(s). Chromosome conformation capture approaches use formaldehyde to cross-link DNA inside cells. Cells are then lysed, followed by enzymatic digestion of the DNA, ligation of the cross-linked fragments, and finally removal of the cross-links, ultimately leaving linked DNA fragments that were in close physical proximity to each other. **Panel D:** CRISPR spacer acquisition as a tool for HGT detection. Using an engineered bacterial strain containing a plasmid with the *cas1-cas2* operon, the sequences of DNA entering the cell are captured and incorporated into the CRISPR array. By sequencing the CRISPR array, HGT events can be observed, including the order in which they occurred.

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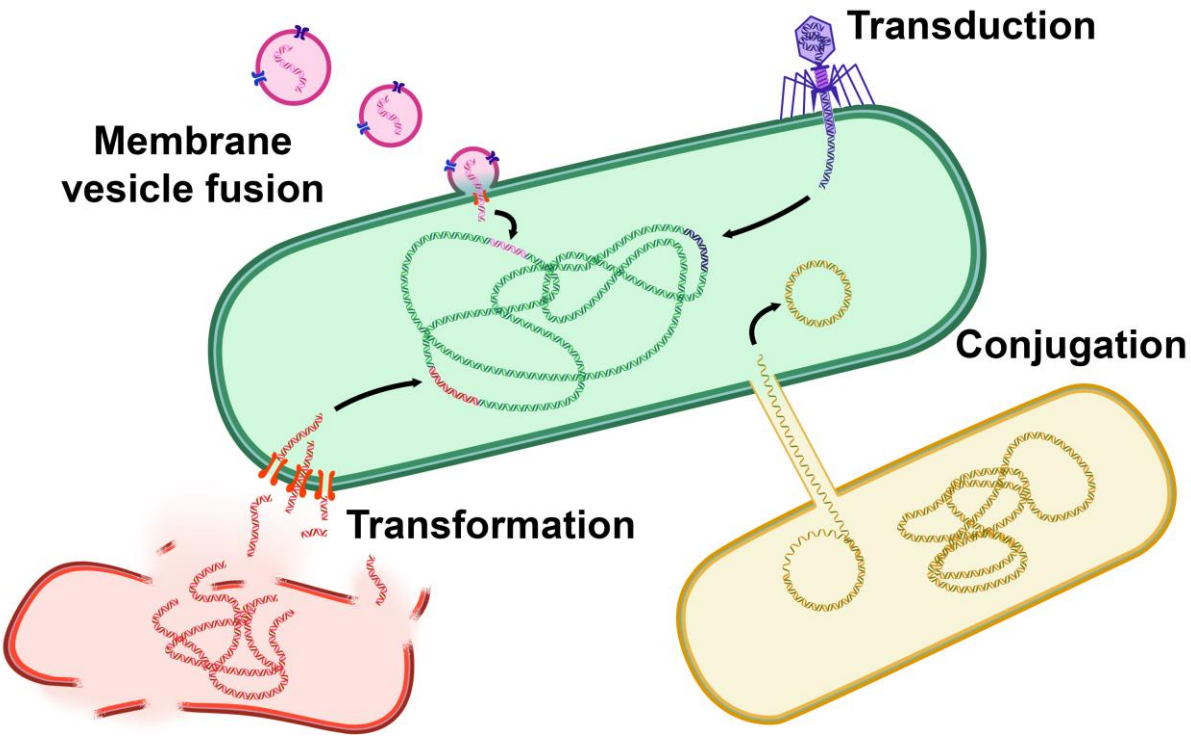
674 **Figure 1**



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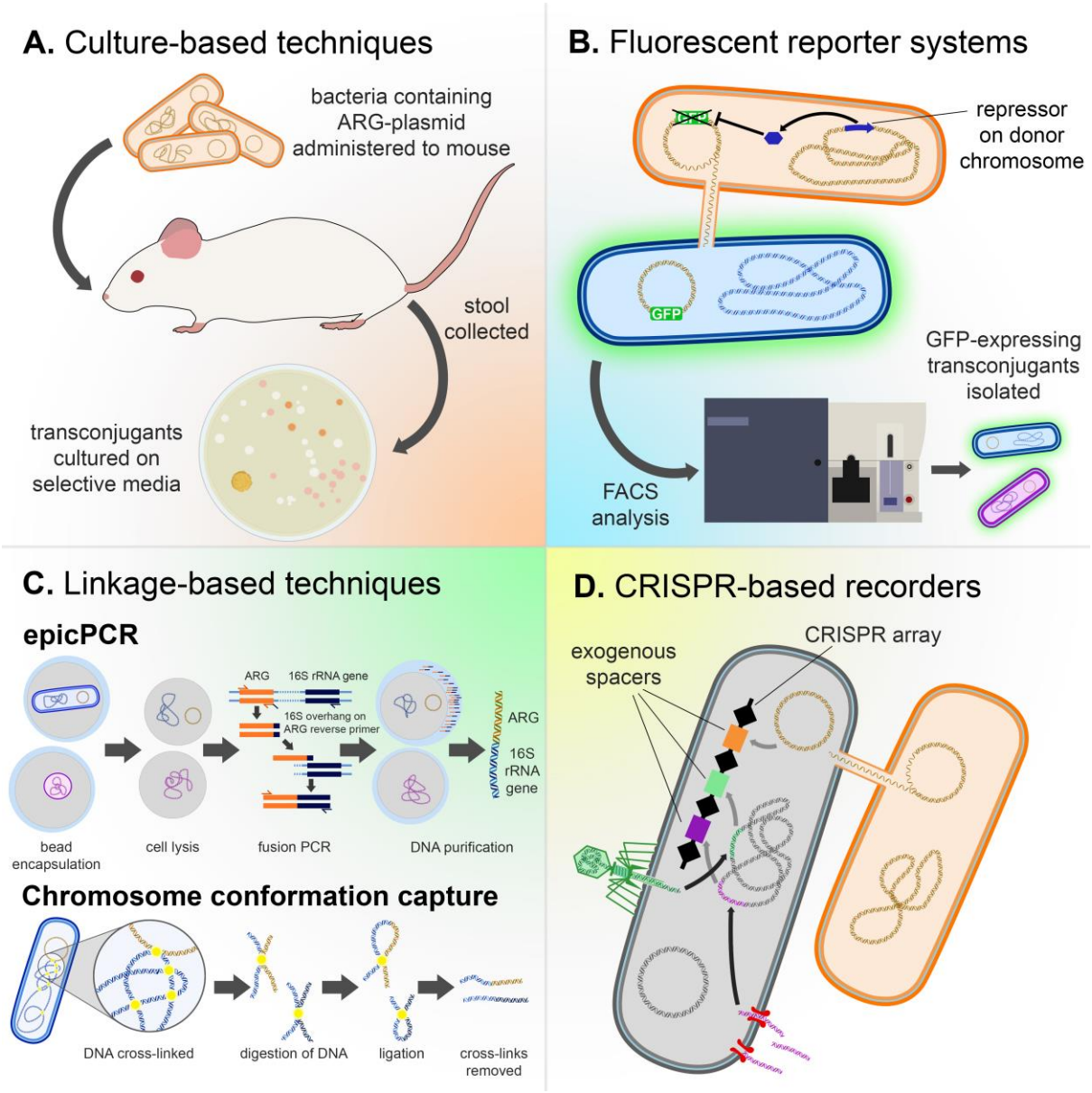
677 **Figure 2**



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