Evolutionary, ecological and biotechnological perspectives on plasmids resident in the human gut mobile metagenome

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Abbreviations: BSH, bile salt hydrolase; FACS, fluorescence associated cell sorting; FISH, fluorescent in situ hybridization; GIT, gastrointestinal tract; HGT, horizontal gene transfer; LAB, lactic acid bacteria; MGE, mobile genetic elements; RING-FISH, recognition of individual genes-FISH; TRACA, transposon aided capture

Numerous mobile genetic elements (MGE) are associated with the human gut microbiota and collectively referred to as the gut mobile metagenome. The role of this flexible gene pool in development and functioning of the gut microbial community remains largely unexplored, yet recent evidence suggests that at least some MGE comprising this fraction of the gut microbiome reflect the co-evolution of host and microbe in the gastro-intestinal tract. In conjunction, the high level of novel gene content typical of MGE coupled with their predicted high diversity, suggests that the mobile metagenome constitutes an immense and largely unexplored gene-space likely to encode many novel activities with potential biotechnological or pharmaceutical value, as well as being important to the development and functioning of the gut microbiota. Of the various types of MGE that comprise the gut mobile metagenome, plasmids are of particular importance since these elements are often capable of autonomous transfer between disparate bacterial species, and are known to encode accessory functions that increase bacterial fitness in a given environment facilitating bacterial adaptation. In this article current knowledge regarding plasmids resident in the human gut mobile metagenome is reviewed, and available strategies to access and characterize this portion of the gut microbiome are described. The relative merits of these methods and their present as well as prospective impact on our understanding of the human gut microbiota is discussed.

Introduction

Higher eukaryotic organisms have evolved in a biosphere dominated by prokaryotic cells (estimated to number -10^30), and live in intimate association with prokaryotic microbes that colonise various body sites to form host-associated ecosystems. Such communities develop and evolve under complex selective pressures, and unlike microbial ecosystems that reside in environments such as the soil and sea, host-associated ecosystems are not only subject to pressures resulting from competition between constituent members, but are also subject to selective pressure at the level of the metazoan host through effects on host fitness.

The co-evolution of host and microbe in this context and the general complexity of many microbial ecosystems frequently results in the development of host-associated microbial communities with emergent properties that directly benefit the metazoan host. Modern humans are no exception, and we play host to numerous microbial communities with the most densely populated being found in the gastrointestinal tract (GIT). The adult human gut microbiota reaches population densities of ~10^{12}–10^{13} individual cells in the distal colon which are dominated by bacteria derived from an estimated 150–800 species (with ~70–80% presently being uncultured). These belong primarily to the Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria divisions, with Bacteroidetes and Firmicutes co-dominating in this community.

This microbial ecosystem plays an intimate role in the development and well-being of the human host, and undertakes a range of functions from which we benefit, including the salvage of energy from the diet through fermentation of components recalcitrant to host digestive mechanisms, protection against pathogens through competitive exclusion, and maturation of the immune system. Conversely, bacterial residents of the gut obtain many benefits from the human host such as nutrients (in the form of mucus and dietary components), as well as a relatively stable environment. Recently, detailed investigation of the mechanisms underlying host-microbe interactions in key cultivable members of the gut microbiota, as well as metagenomic approaches which permit access to the wider uncultivated fraction of this community, have further illuminated the intimate association of host and microbe in the GIT. This includes the ability of gut microbes to direct the development of aspects of host physiology, modulate host immune responses,
Overall, there is an increase in concentrations of virus-like particles, predominantly bacteriophage, observed in gut microbiomes. This has resulted in the development of new ecological and evolutionary models to describe, explain, and predict the co-evolution of host and microbe.

This understanding has not only made the gut microbiota and other human microbiomes the focus of a significant global research effort, but has also prompted many researchers to begin viewing humans and other animals as gestalt entities composed of both prokaryotic and eukaryotic cells, which engage in a complex network of interactions. The role of this flexible gene pool in the development and functioning of the gut microbial community remains largely unexplored, yet there is emerging evidence that the gut mobile metagenome also reflects the co-evolution of bacteria with the human host in the GIT. Some MGE may therefore be unique to or enriched within this ecosystem (Table 1). Therefore, the human gut mobile metagenome is likely to encode genes important for survival and persistence in the GIT as well as activities involved in host-microbe and microbe-microbe interactions in this community.

Furthermore, the high level of novel gene content typical of MGE, coupled with their predicted high level of diversity, suggests that the mobile metagenome constitutes an immense gene-space likely to encode many novel activities relevant to development and functioning of the gut microbiota, and with potential biotechnological or pharmaceutical value. Current estimates predict the diversity of MGE to be exceedingly high, with phage alone estimated to outnumber bacteria by an order of magnitude in many environments, and by around 2–5 times in the human gut microbiota. As such, this vast genetic resource likely accounts for a significant proportion of the “biological dark matter” believed to be extant in the biosphere, yet ranging from marine to terrestrial environments.

### Table 1. Evidence for co-evolution of gut-associated MGE with the human host and the role of the gut mobile metagenome in community function, development and host-microbe interaction

| Study                     | MGE examined                  | Summary                                                                 |
|---------------------------|-------------------------------|------------------------------------------------------------------------|
| Kurokawa et al. 2007      | Conjugal transposons          | CTn-1549 like family of elements designated CTnRINT observed to be enriched in the gut microbiomes of Japanese and American individuals examined. In conjunction a high level of recombinases and integrases also observed in gut microbiomes. |
| Jones et al. 2010         | Plasmids                      | Plasmids or plasmid families enriched and potentially unique to the human gut microbiome identified, with homologous sequences detected in gut microbiomes of geographically isolated hosts (America, Europe, Japan). Enrichment of some functions encoded by plasmids also observed. In particular plasmid pTRACA22 was noted as widely distributed and potentially unique to the human gut microbiome, with RelBE type toxin-antitoxin addiction modules (TAMs) enriched in terms of relative abundance in human gut microbiomes compared with other data sets examined. |
| Jones 2010                | Plasmids                      | Further evidence for wide distribution of pTRACA22 like plasmids and enrichment of pTRACA22 type RelBE TAMs provided from analysis of the METAHIT data set. Functions of HGT and the gut mobile metagenome considered from host perspective and within the holobionte theory of evolution. |
| Zaneveld et al. 2010      | Plasmids                      | Plasmids infecting gut-associated bacteria exhibit the same patterns of habitat-associated gene convergence observed for bacterial chromosomes. |
| Claesson et al. 2006      | Plasmids                      | Lactobacilli commonly harbor large megaplasmids encoding genes proposed to be involved in adaptation to the gut environment. Genes associated with the probiotic or protective effect of some species also appear to be encoded by megaplasmids. |
| Li et al. 2007             | Plasmids                      | Gain of functional capacity in the Japanese gut microbiome attributed to plasmid-mediated HGT of genes conferring ability to utilize seaweed glycans, from marine Bacteroides sp to gut commensal Bacteroides spp |
| Corr et al. 2007           | HGT, putative plasmids        | Demonstrated large inter-individual variation in gut virome composition, but relative intra-individual temporal stability of gut viromes. Functions associated with a wide range of processes in anaerobic gut bacteria found to also be encoded by gut viromes. A dominance of temperate phages observed and a general lack of predator-prey dynamics in host-virus interactions suggested, which is in contrast to other microbial ecosystems. The latter may indicate host-level selection for a stable gut ecosystem. |
| Hehemann et al. 2010      | Bacteriophage                 | Isolation and characterization of bacteriophage infecting Bacteroides sp GB-124, indicated these phage were specific to the human gut, and carriage in the general population is high. |
| Ebdon et al. 2006          | Bacteriophage                 | Demonstrated large inter-individual variation in gut virome composition, but relative intra-individual temporal stability of gut viromes. Functions associated with a wide range of processes in anaerobic gut bacteria found to also be encoded by gut viromes. A dominance of temperate phages observed and a general lack of predator-prey dynamics in host-virus interactions suggested, which is in contrast to other microbial ecosystems. The latter may indicate host-level selection for a stable gut ecosystem. |
| Reyes et al. 2010          | Bacteriophage/virus-like particles | Distinct increase in concentrations of virus-like particles, predominantly bacteriophage, observed in patients with Crohn’s disease compared with healthy controls. Bacteriophage hypothesized to play a role in the dysbiosis of the gut microbiota observed in Crohn disease by destabilizing the community. |
| Lozupone et al. 2008       | General HGT                   | Habitat associated gene convergence of glycoside hydrolases and glycosyltransferases in gut-associated bacteria and archaea largely generated through HGT. |

*Note: Table adapted from various sources, including Jones et al. 2010, Kurokawa et al. 2007, Zaneveld et al. 2010, and others as referenced.*
we remain relatively ignorant of the true diversity, abundance and functions encoded by many types of MGE,\textsuperscript{30,32,48,51,52} MGE are also well documented to promote and facilitate gene flow between distinct and disparate bacterial species driving bacterial adaptation and evolution, and the development of new functional pathways.\textsuperscript{30,31,54-61} There is increasing appreciation for the role of horizontal gene transfer (HGT) in the development of the gut microbiota, and stabilization of important activities of this community.\textsuperscript{8,20,30,31,59,62} Although this may be achieved through recruitment of member species with overlapping metabolic and functional profiles, HGT in the gut microbiota has been implicated as a major factor in the development of a functionally stable community, underlying the dissemination of gene sets encoding for key community activities to a wide range of community members.\textsuperscript{30,62-65} This is thought to generate functional redundancy among community members, which guards against loss of key activities.\textsuperscript{5,30,31,62} In addition, the observation that MGE can facilitate the transfer of genetic material across kingdom boundaries,\textsuperscript{66-68} including transfer from bacteria to mammalian cells,\textsuperscript{69} is of particular significance to host-associated microbial ecosystems such as the human gut microbiota.

Of the numerous types of MGE that will comprise the mobile metagenome of a microbial community, plasmids are of particular significance. Plasmids are frequently capable of mediating their own transfer between distinct species of bacteria, and often encode a range of accessory functions advantageous to their bacterial hosts under particular environmental conditions,\textsuperscript{30-33,52,54-58}; they can also act as vehicles for the dissemination of other MGE such as transposons and integrons.\textsuperscript{30,32,58,70,71} In addition, natural plasmids often form the basis for the development of new molecular tools for organisms lacking basic genetic systems for their manipulation and functional dissection. Ultimately the development of strategies and detailed elucidation of mechanisms underlying host-microbe interactions in the gut microbiota will require the development of molecular tools which permit the detailed characterization of species. Plasmids and other MGE associated with the gut microbiota will likely provide a source of raw genetic material to develop such tools, as has been described for a range of species recently.\textsuperscript{54,72-76} In this article current knowledge regarding plasmids resident in the human gut mobile metagenome is reviewed, and available strategies to access and characterize this portion of the gut microbiome are discussed.

**Overview of Plasmids Associated with Gut Microbes and Encoded Functions**

Plasmid carriage is prevalent among cultivatable members of the gut microbiota, which until recently constituted the only accessible fraction of this community.\textsuperscript{36-44,64,70,72,74-90} Members of the Bacteroidetes, Enterobacteriacea and lactobacilli are frequently found to harbor multiple elements within the same cell,\textsuperscript{40-44,64,72,74,77-84,89} while plasmid carriage by isolates of common gut-associated Bifidobacterial species has generally been reported to be less common, with plasmid-free isolates encountered at a higher frequency.\textsuperscript{79,85-88} In contrast, there is a general paucity of data regarding plasmid carriage by numerically dominant members of the gut microbiota belonging to the Firmicutes division (predominantly Clostridia), which co-dominate with members of the Bacteroidetes division in this ecosystem.\textsuperscript{16-18} The general lack of data regarding clostridial plasmids from this population is likely a reflection of the difficulty in culturing the relevant members of gut Firmicutes, as well as limitations of classical endogenous and exogenous methods for plasmid isolation (discussed in detail in subsequent sections).\textsuperscript{31,53-58}

More recently, genomic and metagenomic analyses have been adapted for study of gut-associated plasmids and have provided evidence for the co-evolution of plasmids infecting members of the gut microbiota with the human host (summarized in Table 1).\textsuperscript{30,32,49,50} In particular, Zanveld et al. (2010) have demonstrated that in the gut-associated bacterial species they analyzed, the gene content of plasmids exhibited the same general patterns of habitat-associated gene convergence found in the corresponding bacterial chromosomes.\textsuperscript{49} Closely related bacteria exhibited low levels of inter-species gene convergence moving toward greater levels in both chromosomes and plasmids with increasing phylogenetic distance.\textsuperscript{49} This is proposed to demonstrate increased specialization among closely related species (likely driven by competition for similar resources), but long-term convergence of functions at higher phylogenetic levels (indicative of host level selection).\textsuperscript{5,30,49} In essence, these findings and those summarized in Table 1, support the hypothesis that plasmids, and the mobile metagenome in general, are likely to encode functions symptomatic of challenges and activities important to life in the human gut.\textsuperscript{30-35}. These findings also predict the existence of plasmid-encoded functions, or plasmid families, that are enriched or unique to the gut microbiota. Evidence for this has recently been provided in a metagenomic-based analysis of plasmids and encoded functions in the gut microbiome.\textsuperscript{32} In this study, plasmid pTRACA22 was identified as potentially unique or enriched in the gut mobile metagenome, when compared with murine or environmental metagenomic data sets.\textsuperscript{32} Sequences with high homology to pTRACA22 were detected in metagenomic datasets derived from the gut microbiomes of geographically isolated individuals indicating a long-term association with this community, and were not detected in any environmental or murine data sets analyzed.\textsuperscript{32} Furthermore, some functions encoded by the pTRACA22 element were found to be enriched in the human gut microbiome relative to other environments, including a novel RelBE type toxin-antitoxin addiction module.\textsuperscript{32}

The enriched pTRACA22 RelBE module has now also been confirmed to be a functional and expressed gene system in the surrogate \textit{E. coli} host (by RT-PCR), and preliminary PCR surveys with primers specific to the pTRACA22 RelBE genes has indicated that this module may be present on a large proportion of plasmids isolated from the gut environment (28/42 plasmids tested; B. Jones, unpublished data). Although it is presently unclear what role these pTRACA22 type RelBE modules may play in the gut microbiota (if any), they have been associated with numerous functions in bacteria relevant to life in the GIT, including survival under adverse conditions and exposure to antibiotics, resistance to bacteriophage predation, regulation of
gene expression, and effects on human cells.\textsuperscript{30,32,91-101} This highlights how analysis of the mobile metagenome can identify novel functions with the potential to be important in community function and development, as well as access to activities of potential biotechnological or pharmaceutical or clinical interest. However, studies characterizing plasmid-encoded functions in the human gut microbiome have typically focused on clinically relevant traits relevant to treatment or pathogenesis of infectious disease, but factors mediating survival and persistence among gut commensals are now receiving more attention.

**Antibiotic resistance.** Many studies of plasmids and other MGE resident in cultivable members of the gut microbiota have focused on those conferring antibiotic resistance, and harbored by easily cultivated members of this community that constitute prominent and problematic opportunistic pathogens, such as strains of *E. coli*, Enterococci, *Klebsiella pneumonia* and Bacteroides spp. The human gut is generally regarded to be a reservoir for antibiotic resistance genes\textsuperscript{36,39,89} exemplified by the observed prevalence of genes encoding tetracycline resistance in human gut isolates of Bacteroides species even before the widespread introduction of this antibiotic.\textsuperscript{36} Furthermore, recent metagenomic analyses have highlighted the potential diversity of resistance determinants that may be encoded by the gut microbiota, and their frequent association with putative MGE.\textsuperscript{39}

Plasmids encoding resistance to a wide range of antibiotics have been identified in members of the human gut microbiota, including those conferring resistance to β-lactams, sulphonamides, tetracycline, chloramphenicol, trimethoprim, kanamycin, erythromycin, streptomycin, clindamycin, and metronidazole.\textsuperscript{38,79,80,82,84,90,102-115} Such plasmids are also frequently mobile and autonomous transfer between members of the mammalian gut community, as well as between commensal organisms and species considered to be transient colonizers, has been demonstrated both in vitro and in vivo.\textsuperscript{38,81,82,84,90,102-105,107-109,113-115} Although the majority of studies have been justifiably concerned with the spread of plasmid-encoded antibiotic resistance between common and pathogenic species associated with the gut microbiota, there is also increasing interest in understanding if less well characterized members of the gut microbiota also serve as reservoirs of mobilizable antibiotic resistance determinants, and in particular gut-associated species considered beneficial to human health.

The carriage and transfer of plasmids between bacteria in the GIT, such as Bifidobacteria and lactobacilli spp, is of considerable interest since members of these groups are not only part of the normal gut microbiota, but also important industrial organisms used in the manufacture of many fermented dairy products, as well as being formulated as probiotics.\textsuperscript{40,41,64,102,103} Plasmids are considered to be common among some groups of Lactic acid bacteria (LAB) and have been shown to encode a wide range of traits that facilitate survival and adaptation to the gut environment.\textsuperscript{40,41,64,74,75,85,106,114,116-119} Numerous studies have also reported antibiotic resistance among isolates of these organisms, including those obtained from food products, the human GIT, and used as probiotics, and there is increasing interest in the mobility of the relevant genes.\textsuperscript{102,106,114,115,120-125}

Several studies have demonstrated the presence of antibiotic resistance genes encoded by plasmids and other MGE in species of LAB, and highlighted the transfer of both recombinant and natural plasmids to other members of the gut microbiota.\textsuperscript{102,103,110,114,115,126-128} Further research is still required, however, to enhance our understanding of the potential for this important group of gut related bacterial species to harbor antibiotic resistance genes, and transmit these to other members of the gut microbiota, as well as transiently colonizing pathogens. The latter scenario is of particular concern both in terms of patient welfare and escalating levels of resistance among prominent opportunistic pathogens (which include many members of the normal commensal gut microbiota), and the increasing long-term usage of probiotics by the general public.

**Adaptation, survival and persistence in the gut environment.** While the role of plasmids in the dissemination of antibiotic resistance among clinically relevant gut-associated bacterial species has received much attention, numerous other accessory functions may be encoded by plasmids, and there is clear potential for plasmid-encoded traits to contribute to the development and functioning of the gut microbiota.\textsuperscript{30-32,50,89} Activities relevant to aspects of community function and host health, including utilization of diverse nutrient sources and degradation of xenobiotic compounds,\textsuperscript{31,40,41,64,116,118} virulence factors,\textsuperscript{79,129-139} bacteriocins,\textsuperscript{141-145,177,179,140,142} and adhesion to host epithelial cells,\textsuperscript{43,78,90,129,130,136,138} have all been identified on plasmids from gut-associated species. In a number of instances, plasmids encoding multiple functions that increase fitness in the GIT have been isolated and characterized.\textsuperscript{31,40,44,64,78,131,133,137} Many of these traits are of potential pharmaceutical, or biotechnological value, for example in the discovery of novel bioactive compounds or the rational design of more effective probiotics, including probiotic-based platforms for the delivery of drugs, vaccines, or other therapeutic interventions.

Lactobacilli also provide excellent examples of such plasmid-mediated adaptation to the GIT, and biotechnologically relevant plasmid-encoded traits. In particular, recent studies have highlighted the frequent carriage of large megaplasmids by species of lactobacilli isolated from human and animal sources, including numerous strains obtained from faeces or other intestinal samples.\textsuperscript{40-42} These include the probiotic strain *L. salivarius* UCC118, which has a genome composed of multiple replicons, including a large 242 Kb megaplasmid designated pMP118.\textsuperscript{42} The UCC118 megaplasmid encodes multiple traits relevant to the survival of this species in the GIT, including tolerance to bile, bacteriocin production, and contingency pathways for cell wall biosynthesis and redox balance.\textsuperscript{42}

Collectively these functions are proposed to endow *L. salivarius* UCC118 with a repertoire of accessory traits or pathways that permit a high degree of genetic and phenotypic flexibility, which facilitates survival in the gut environment by enhancing ability to adapt to transient ecological niches that arise in this community, tolerate fluctuations in diet and community structure, and overcome intrinsic barriers to colonization of the GIT.\textsuperscript{42} Functions encoded by the pMP118 megaplasmid are also involved in the “probiotic effect” of UCC118, and the pMP118
encoded bacteriocin system has been demonstrated to underlie the ability of UCC118 to protect mice against infection by the foodborne pathogen *Listeria monocytogenes.* Overall, the lactobacilli megaplasmids serve as an important example of the functions that may be encoded by the gut mobile metagenome and how these can influence community development and impact on host health. Such traits are also of considerable interest in the rational design of more effective probiotics, and probiotic-mediated platforms for therapeutic or prophylactic disease intervention.

Bacteriocins with activity against prominent foodborne pathogens have also been found to be encoded by plasmids harbored by a range of other gut-associated microorganisms, such as *E. coli, Pediococcus acidilactici* and *Bifidobacterium infantis.* In light of the rising levels of antibiotic resistance and the relative dearth of programs aimed at developing new antibiotics, alternative strategies to control or prevent infections are of significant interest, and therefore bacteriocins have potential biotechnological value. This highlights the pharmaceutical potential of the gut mobile metagenome, and illustrates how this sphere of the gut microbiome may also be a source of novel bioactive agents with potential commercial and clinical applications.

Functions relevant to colonization of the gut and survival in this environment have been attributed to plasmid-encoded genes in a number of species. These include mechanisms which promote adherence to host tissues, as described for the human gut commensal *E. coli* SE11 which harbors six plasmids encoding genes involved in bacteriocin production, tetracycline resistance as well as adherence to host cells. In the case of attachment to host tissues it has been proposed that this may be a feature of conjugative plasmids and transposons in general. Pili and associated conjugation machinery assembled by these MGE, in order to transfer plasmids, may also directly facilitate bacterial adhesion to human cells and tissues. In this scenario, harboring conjugative elements may be better able to colonise the GIT which would serve not only to enhance the fitness of the host organism, but also offset the fitness cost of plasmid carriage by promoting maintenance of plasmid harboring cells, and by default maintaining the plasmids they carry within the gut community.

Other plasmid-encoded colonization factors may include those that mitigate the toxic effects of bile acids. Thus, the observation that bile tolerance or resistance mechanisms are encoded by plasmids in some gut-associated species is also of considerable interest. Conjugated bile acids are key components of bile, and have diverse functions in the host ranging from absorption of dietary lipids to roles as key signaling molecules regulating a variety of systemic metabolic processes. These compounds are also toxic to bacteria and constitute a significant barrier to colonization of the GIT. Genes encoding bile salt hydrolase (BSH) activity, which catalyzes the de-conjugation of bile acids, and has been shown to facilitate tolerance to these compounds in a range of gut-associated bacterial species. This activity is widely distributed in the gut community and present in bacterial species from all major phylogenetic divisions, as well as gut associated archael species. Such wide dissemination provides a high level of functional redundancy for this activity within the community and it seems likely that HGT has contributed significantly to its broad distribution, with evidence for the transfer of relevant genes between distinct domains of life (Bacteria and Archaea) also documented. However, the role of bile acids as signaling molecules also opens the possibility for microbial BSH activity to influence wider aspects of host physiology through perturbations in bile acid signaling via production of modified bile acid derivatives that display altered binding characteristics for relevant receptors. The observation that this function is also encoded in the mobile metagenome of the gut microbiota suggests further exploration of plasmids and other MGE associated with this community will yield important fundamental insights into its development and overall function, as well as mechanisms by which gut bacteria interact with the human host.

**Virulence, commensalism and lifestyle diversification.** Plasmids and other MGE comprising the gut mobile metagenome may also encode traits involved in virulence and pathogenesis, and a number of members of the normal microbiota in healthy individuals are also prominent opportunistic pathogens. A wide range of gut-associated pathogenic species have been reported to harbor plasmids which confer the ability to produce key virulence factors or other traits contributing to disease processes. Plasmids coding for the production of toxins and virulence factors, cell invasion and survival within macrophages, attachment to host tissues, immune evasion, type III secretion systems and associated effectors, as well as host-microbe signaling processes relevant to pathogenesis, have all been described in gut-associated pathogens. *E. coli* has been particularly well studied in this regard, and in the case of gut-associated *E. coli* strains, plasmids encoding a wide range of traits appear to have played a major role in the diversification of lifestyles adopted by this organism, which range from beneficial gut commensal to highly virulent primary pathogen. Of the six intestinal *E. coli* pathotypes, all possess virulence plasmids and HGT in general has been pivotal to the emergence of a pathogenic lifestyle in these strains. A key example is O157:H7 in which several of the major virulence factors have been acquired by HGT, such as the shiga toxin via bacteriophage, while plasmids encoding hemolysins, toxins and factors mediating attachment to host cells are also common. In particular, plasmid pO157 is reported to be present in around 99–100% of human O157 clinical isolates, and highly conserved, differing only by single nucleotide polymorphisms between many isolates. Conversely, beneficial or harmless strains of gut-associated *E. coli* also appear to frequently harbor plasmids, including those encoding functions which facilitate their commensal lifestyle, many of which are also functions associated with virulence plasmids, such as attachment to host tissues. Although the presence of plasmids or other MGE encoding potential virulence attributes among members of the normal gut microbiota in healthy individuals may initially appear counter-intuitive, a wide variety of mechanisms for host-microbe
interaction may be of utility to both commensals and pathogens. In particular, mechanisms that contribute to colonization of and persistence within the human GIT, as well as host-microbe signaling and immune modulation, may be put to use by both beneficial species and pathogens alike, albeit with radically different outcomes for the host. The deployment of the same basic machinery for host cell adhesion by both commensal and pathogenic *E. coli* species in this ecosystem is a prime example, and mechanisms for adherence to host cells and colonization of the gut epithelium, have been shown to be plasmid-encoded in both pathogenic and commensal strains. This highlights the potential for the patho-biotechnological exploitation of the gut mobile metagenome in the rational design of probiotics and associated delivery systems.

Overall, the involvement of plasmids as well as other MGE in diversification of lifestyles among distinct strains of the same species colonising the gut environment, highlights the potential for MGE to provide bacteria with a flexible and diverse genetic resource that permits adaptation to a wide range of ecological niches within a complex microbial community, and ultimately enhances the fitness and success of the species as a whole. There is also the possibility to extend this basic concept to the gut microbiome, with the mobile metagenome facilitating lifestyle diversification and adaptation of constituent members, generating functional redundancy, and promoting stability. This would enhance the overall fitness not only of the entire community, but also of the associated metazoan host by virtue of generating a stable and productive gut microbiota with associated benefits. Such hypotheses fit well with the proposed hologenome theory of evolution, but at present there is little evidence to confirm or refute their accuracy.

The mobility of virulence factors among gut pathogens also has important implications for the evolution of deleterious host-microbe interactions in this ecosystem. It is currently unclear if plasmids resident in the gut mobile metagenome are an important reservoir of potential virulence attributes which may be accessed by emerging gut pathogens or transiently colonizing species, but the clear contribution of HGT and plasmid-encoded factors to the evolution of virulence in a wide range of prominent gut pathogens argues for further investigation of the human gut mobile metagenome from this perspective.

Plasmid Transfer in the GIT

In light of the increasing burden of antibiotic resistance in bacteria, and the growing appreciation of the gut microbiota and other human associated microorganisms as reservoirs for antibiotic resistance genes, understanding the factors influencing HGT in environments such as the GIT are of considerable importance from this perspective alone. However, such information is also required to complete our fundamental understanding of how the gut microbiota develops and functions, and to access the full biotechnological and clinical potential of this microbial ecosystem. The mammalian GIT is considered a hotspot for HGT, likely due to a high population density coupled with the presence of large numbers of closely related species derived from relatively few phylogenetic divisions, factors known to be beneficial to conjugal transfer of plasmids. In addition, the growth of bacteria as biofilm-like communities associated with the intestinal mucus layers may also promote the transfer of plasmids between constituent species, as well as between indigenous members and transiently colonizing species.

The in vivo transfer of plasmids in the GITs of mammalian or avian model organisms has been demonstrated between both closely related species, as well as between more distantly related phyla. These include both Gram-negative and Gram-positive species derived from all major phylogenetic divisions (Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria) of the human microbiota, but most studies have traditionally focused on the transfer of antibiotic resistance genes among potentially pathogenic species that frequently form part of the normal human gut bacterial community. Examples include the transfer of plasmids encoding resistance to ampicillin between distinct strains of *E. coli* in the infant GIT, the acquisition of plasmids encoding carbapenem resistance by commensal *E. coli* from *K. pneumonia* strains, and the transfer of plasmids encoding erythromycin resistance from probiotic lactobacilli species to *E. faecalis*. Of particular concern are recent reports detailing the transfer of plasmids harboring extended spectrum β-lactamases among gut-associated species, which not only confer resistance to a wide range of commonly used β-lactam antibiotics including those designed to resist β-lactamase activity, but are also associated with multiple antibiotic resistance phenotypes. The broader range transfer of plasmids between Gram-negative and Gram-positive members of the gut microbiota has also been demonstrated, and recent studies have highlighted how some plasmids found in gut-associated species may function as novel broad range shuttle vectors, and the potential for these to develop naturally in the mobile metagenome.

Factors influencing plasmid transfer rates. Numerous factors have been found to contribute to the relative rates of transmission for different plasmids. These include attributes of a particular plasmid and recipient/donor species studied, such as the conjugation machinery employed, the physiological status of donor and recipient cells, compatibility of plasmids with host cell replication machinery and potentially other plasmids already harbored by the cell. Collectively these factors will dictate the host range of a particular plasmid and therefore how widely it may be disseminated within a microbial community with a given population structure.

Furthermore, intrinsic features of the plasmid, such as its encoded functions and the level of selection for these, will also influence transfer rates and stability in the GIT. For example, plasmids producing short rigid pili have been observed to transfer more efficiently when donor and recipient cells are associated with a solid surface, while those that utilize long flexible pili can transfer equally well in liquid culture. In terms of selection and the influence of plasmid-encoded traits, it is well documented that plasmid transfer is generally higher when there is strong positive pressure for traits encoded, as exemplified by studies of plasmids harboring mercury resistance in soil bacteria.
and indicating some plasmids may have an optimal level of selection at which transfer rates are highest, as opposed to a simple cumulative effect of increasing selective pressure.  

In addition, ability to transfer will also be impacted by aspects of donor and recipient cell abundance, their physiological status, and distribution in this environment. In terms of host cell status, the availability of nutrients, growth phase and general cell health are all implicated in the kinetics of plasmid transfer, and therefore the relative fitness of donors and recipients within the gut environment will also play a role in the levels of plasmid transfer observed in vivo. However, much accepted wisdom regarding factors affecting plasmid transfer, and the degree to which these influence rates of conjugal transfer, has been derived from laboratory experiments using batch cultures.

Although in vitro systems have provided much insight into the kinetics of plasmid transfer, these effectively represent artificial and simplistic model systems which do not incorporate, or permit assessment of, key parameters that are likely to significantly influence plasmid transfer in vivo. As well as factors such as population density of donor and recipient cells, and their relative status within a population (e.g., well adapted allochthonous members of the community vs. transiently colonizing autochthonous species); the complexity of the associated microbial ecosystem, the motility of host and donor species within the environment, the presence of homogenizing mechanical forces or flow, and physicochemical characteristics of the environment can also impact greatly on the observed rate of transfer.

**Factors influencing plasmid transfer in the GI tract.** While some studies of plasmid transfer in natural environments, including the gut, have shown a lower rate in vivo than in vitro, a growing number of studies investigating transfer in the GIT using animal models have described a generally higher rate of transfer for certain plasmids in vivo, compared with in vitro laboratory conditions, and indicate existence of indigenous transfer promoting factors in the GIT. Furthermore, in vitro assays have been shown to underestimate the true rates of transfer in vivo when factors other than donor and recipient cell densities are accounted for; however, it should also be noted that many studies investigating plasmid transfer in vivo have used gnotobiotic animals which lack a gut microbiota, or models in which the microbiota has been disrupted using antibiotics to permit colonization by donor or recipients (reviewed in ref. 90). As the presence of a complex microbiota may itself be a factor limiting the rate of conjugal transfer in the GIT, experiments in germ free animals likely do not reflect the true rate of plasmid transfer in this environment. Nevertheless, a growing number of studies support the GIT as a hotspot for HGT, and suggest that certain features of this environment may enhance plasmid transfer.

A particular feature of the GIT that is thought to provide favorable conditions for plasmid transfer is the intestinal mucous layer, and the formation of biofilm-like communities within this matrix. Plasmid transfer is thought to be enhanced by the close cell-cell contacts generated when bacterial biofilms are formed, and rates of transfer for some plasmids are observed to be higher in biofilm communities compared with planktonic cell populations, including studies with gut-associated species. Licht et al. (1999) indicated that in biofilms the initial spread of plasmids after introduction of donor cells is rapid, but subsequently reduces to very low levels or ceases completely. This is believed to be due to a combination of fixed spatial positions of donor and recipient cells within the biofilm, and the general limitation of plasmid spread to cells immediately surrounding donors during initial transfer events, rather than continuous rounds of subsequent transfer initiated by newly formed transconjugants.

The observed lack of subsequent transfer from new transconjugants is most likely a result of the physiological status of cells within the biofilm, as well as variation in nutrient availability in different sections of the biofilm. The authors also noted that the transfer kinetics of plasmids in vivo were more similar to those observed in biofilm communities than in mixed liquid cultures, which was attributed to growth and transfer within the mucus layer. The observation that conjugative plasmids enhance biofilm formation in vitro on abiotic surfaces by virtue of their transfer machinery, which may also promote adhesion to mucosal surfaces, reinforces the mucosa associated plasmid transfer hypothesis.

Importantly, this model makes certain predictions regarding the ability of plasmids to transfer after initial colonization of the mucus layer, and suggests that this feature of the GIT will enhance the initial transfer of plasmids from donor cells to recipients, but subsequent transfer will be limited by the nature and physiological status of surrounding cells. If so, then transfer of plasmids to donor cells would be expected to not only occur rapidly after initial colonization of the intestinal tract, but depend greatly on the nature of surrounding cells and the numbers of suitable recipients in the gut community. In contrast, the proposed rapid nature of initial transfer events increases the likelihood of plasmid transfer from transiently colonizing species to normal members of the gut microbiota. Aside from the acquisition of undesirable traits such as antibiotic resistance, this would also afford the gut microbiota greater opportunity to sample the wider flexible prokaryotic gene pool and acquire new functional capacity of benefit to both host and microbe. The recent observation that the Japanese gut microbiota has been subject to functional enhancement through plasmid-mediated HGT fits well with this scenario. In this example, genes required for utilization of seaweed glycans by gut commensals were shown to have been acquired from marine bacteria that naturally colonise dietary seaweeds, and frequently come into contact with gut microbes through the consumption of uncooked seaweed.

Given the ancient nature of the proposed transfer events underpinning this functional adaptation, the exact mechanisms involved cannot be determined with certainty, however, genetic evidence indicates this gene exchange was mediated by plasmid transfer, and it is tempting to speculate that the kinetics of plasmid transfer observed in other studies of the GIT, particularly the potential for rapid initial transfer from incoming foreign
organisms, were important to this event. Regardless of the factors dictating the original transfer event, such studies highlight the potential impact on the host of plasmid transfer in the gut microbiota, in this case mediating access to a new dietary energy source.10

Can host diet influence rates of plasmid transfer in the GI tract? Other features of the GIT are also thought to be favorable for conjugal transfer. In other habitats, such events have been shown to occur at highest rates when cells are in a nutrient rich environment, with nutrient limitation observed to reduce transfer rates.160,179-182 In general the GIT should provide indigenous microbes with an ample and steady supply of nutrients, and this may also be true for some transiently colonizing organisms. However, in the case of allochthonous species the high level of competition extant in the gut microbiota and the resulting occupation and exploitation of available ecological niches by autochthonous species is likely to effectively exclude many such organisms and limit their ability to proliferate and utilize available nutrient sources.2,8,183 Therefore, the proposed advantages of mucosal associated communities mediating rapid transfer of plasmids may be offset by effects of competition and nutrient limitation in transiently colonizing species, reducing transfer between allochthonous and autochthonous species.

In this context it is of note that for certain plasmids, changes in host diet have been found to have a significant impact on rates of plasmid transfer.103,104 While the GIT may provide a relatively steady source of nutrients overall, natural variation in diet means this is unlikely to be completely consistent, and when considered in terms of the complexity and niche specialization that exists in this community, it seems unlikely that all members will receive a steady and constant supply of readily utilisable nutrients. Furthermore, although in industrialized countries access to food supplies on demand are taken for granted, a significant portion of the extant human population is subject to periods of starvation.

The influence of diet on plasmid transfer could also occur via alternative mechanisms, not least of which is the impact of diet on the physicochemical properties of the intestinal milieu. In addition to factors such as nutrient availability, the fluidity of intestinal contents, the production of metabolites by indigenous community members, effects on community structure, and gut motility, are all factors that may be potentially influenced by diet, and are believed to influence plasmid transfer.89,155,157 There is also potential for diet to influence rates of plasmid transfer by modulating the level of host digestive secretions in the intestinal lumen. A prime example of this is the increased levels of bile secreted in response to diets high in fat. Exposure to bile has been found to increase the rates of curing and plasmid loss in Salmonella enterica, for otherwise highly stable plasmids,185 and the bile salt deoxycholate was subsequently shown to reduce the transfer rates of the virulence plasmid pLST in vitro.138

For E. coli, formation of the conjugation pilus associated with transfer of the F plasmid, has been shown to increase sensitivity of this organism to bile salts commonly encountered in the human GIT, particularly conjugated bile acids.186 In this case, the increased sensitivity of pili expressing cells would likely serve to destabilize plasmids in gut dwelling populations in compartments where levels of bile acids are high (such as the duodenum, jejunum and proximal ileum), and restrict transfer to regions of the GIT where bile levels are low, such as the distal ileum and colon. Of particular interest in this context are the observations by Tuohy et al. (2002), who documented a decreased level of trans-conjugant formation when rats were fed a high fat human diet.103 Although the mechanisms involved in this dietary induced change in plasmid transfer were not elucidated in this study, given that increased fat intake increases levels of bile in the intestine it is possible that this impact of diet on intestinal physiology was involved in the observed reduction in plasmid transfer. Overall, the effects of diet on plasmid transfer merit further investigation, and may provide options for suppressing in vivo transfer among certain groups of bacteria in the human GIT.

This also highlights the potential variation in plasmid transfer rates across different compartments of the GIT, which vary in terms of parameters known to influence plasmid transfer.138 Few studies account for the distinct compartmentalization of the GI tract and the variation in environmental parameters along the length of the intestinal tract such as pH, osmolarity, oxygen tension, and metabolic diversity, which are encountered by microbes from entry to exit. It is likely that the changing environmental factors encountered along the length of the GIT add an additional level of complexity to plasmid transfer in vivo. Plasmid transfer rates at different locations in the GIT have yet to be explored in detail, with the majority of in vivo studies concerned only with the levels of trans-conjugants that appear in faeces. However, evidence for variable plasmid transfer rates among different compartments of the GIT may be exemplified by the impact of bile on the transfer of Salmonella virulence plasmids,138 as well as the potential role of this endogenous host secretion in plasmid curing in this prominent gut pathogen.185,186

Phage-mediated transfer of plasmids. There also exists the potential for plasmid transfer in the gut to occur through mechanisms other than conjugal transfer. In particular, the transfer of plasmids between Lactococcus lactis and Streptococcus thermophilus through phage-mediated transduction has recently been demonstrated.187 This adds a further possible mechanism of plasmid transfer relevant to the GIT, the significance of which is yet to be explored in terms of HGT in the gut microbiota and microbial ecosystems in general. Because bacteriophages are abundant in the gut microbiota, and this community is essentially comprised of high numbers of closely related strains and species, it is conceivable that this mechanism plays an important role in the transfer of some plasmids in this community. However, it seems unlikely that a wide range of plasmids will be compatible with this mechanism of transfer, and given the narrow host range of most phage it is probable that the contribution of this form of plasmid transfer in the GIT will be restricted to a relatively low number of plasmids, and their transduction among closely related strains or species.

Future considerations for the study of plasmid transfer in the GI tract. Overall our understanding of the relative rates of plasmid transfer in the gut are limited by available methods to study these events, which typically utilize artificially introduced selectable recipient strains (often through colonization of either
germ free animals or post antibiotic disruption of the extant microbiota) and subsequent monitoring of trans-conjunct transfere numbers in faeces. In most cases the motivation for such studies has been to develop an understanding of the transfer of antibiotic resistance genes in the gut microbiota, and the ability of this community to acquire the relevant MGE and act as a reservoir of these traits. As such donor and/or recipient species as well as the plasmids tested may not represent species, elements, or functions prevalent in this community.

The functions encoded by a plasmid are likely to be of much significance to the relative in vivo transfer rate, and many studies have demonstrated that transfer occurs more frequently when selective pressure is introduced for plasmid-encoded traits. As such, utilizing plasmids derived from the gut microbiota itself, and encoding functions relevant to environmental stresses normally encountered by well adapted gut inhabitants should provide a more accurate assessment of the rates of plasmid transfer among members of this community under normal conditions. Furthermore, the importance not only of the prevalence of the donor and recipient cells within a community, but also their physiological status upon rates of plasmid transfer, also suggests that utilizing species normally present in this community and well adapted to life in the gut, in conjunction with naturally occurring plasmids infecting these species, will greatly enhance our understanding of the rates of gene transfer in the gut community.

Thus, our knowledge regarding transfer of plasmids and other MGE that naturally form part of the gut mobile metagenome, as well as the functions they encode and how they contribute to host health is generally limited. Recent metagenomic studies providing evidence that HGT is not only prevalent but important in the evolution of this community and its impact on host health and development, highlight the need for a greater understanding of this aspect of the gut microbiota (Table 1). However, a range of methods for plasmid isolation are available and new methodologies have been developed which should permit greater access to the gut mobile metagenome and allow wider study of this important sphere of host-associated microbial ecosystems, including greater access to the biotechnological and pharmaceutical potential of the gut mobile metagenome.

**Strategies to Access Plasmids Resident in the Gut Mobile Metagenome**

Despite the potential contribution of the gut mobile metagenome to the evolution and functioning of the gut microbiota, including its impact on host health, this flexible gene pool remains largely unexplored. Such studies are technically challenging and investigation of MGE associated with microbial communities has traditionally been impeded by the large number of uncultivated species comprising these ecosystems, including the gut microbiota where ~70–80% of species remain uncultured, as well as the enormous diversity of MGE that may be associated with complex microbial ecosystems. Furthermore, the high levels of variability in terms of gene content and architecture inherent to MGE such as plasmids, has precluded the development of universally applicable methods for survey and acquisition of certain MGE types comprising the mobile metagenome of a particular microbial ecosystem.

In contrast to studies of the population structure of microbial communities, in which the species present can be identified through a range of universally applicable culture-independent approaches (most commonly 16S rRNA gene amplification and sequencing-based strategies), no such global census can presently be generated for plasmids or other MGE, and given the malleable nature and mosaic structure of many elements characterized in detail so far, coupled with the lack of regions universally conserved in all plasmids (analogous to phylogenetic anchors such as the 16S rRNA genes in bacteria), it is difficult to envision the development of such strategies with current knowledge and available technologies. As such, studies of plasmids associated with bacterial communities are impeded by a fundamental lack of knowledge regarding the composition of the resident plasmid population in terms of numbers and types of elements present. Concordantly, the development of methods to access these elements is challenging, and evaluation of their ability to acquire a representative cross-section of elements present is virtually impossible.

Although technically challenging, a range of methods has been developed for accessing plasmids resident in microbial communities, which have all produced valuable insights into functions of MGE and the bacterial mobile metagenome. Table 2 summarizes current strategies available for accessing plasmids resident in the gut microbiota and other microbial communities as well as their relative strengths and weaknesses when applied to the study of complex microbial ecosystems such as the gut microbiota. These range from the classical endogenous and exogenous plasmid isolation methods, to more recent metagenomic approaches such as the Transposon Aided Capture System (TRACA) system and the direct detection of plasmid sequences in standard metagenomic libraries.

**Endogenous isolation of plasmids.** The most straightforward and well-established method for plasmid acquisition is the direct extraction of plasmids from the native host species. This typically requires cultivation of the plasmid host species, or a mixed microbial population with or without selective enrichment for traits of interest. Subsequently, isolated plasmids are often transformed into a surrogate host, if possible of the same species, and plasmid-encoded traits assessed through predominantly phenotypic assays. The complete nucleotide sequences of isolated plasmids may also be obtained, and plasmid-encoded functions characterized in silico. The generation of plasmid sequences during whole genome sequencing projects may therefore also be considered as examples of the endogenous approach, for instance the characterization of the *L. salivarius* UCC118 plasmid complement.

A major advantage of this method over other plasmid isolation systems is the unambiguous identification of the natural host species of plasmids isolated. However, since plasmids are often promiscuous and capable of infecting multiple host species, endogenous isolation does not necessarily provide a complete picture of the range of host species in which a particular plasmid may reside. In addition, the culture-dependent nature of this
In these cases, the high proportion of potential augmentation/future plasmids belonging to the same incompatibility group as generally only incomplete, partial plasmids identified. Scope for retrieval of plasmid use of a mixture of Tn elements. Plasmids belonging to the same incompatibility group as currently available Tn elements and surrogate may permit capture of MGE other potentially applicable to all circular culture-independent sequence-based characterization. Initial capture independent of original bacterial host unknown. Potentially capable of isolating range of plasmids isolated dependant on mating cell physiology and plasmid transfer kinetics. As new organisms are isolated and culture-independent sensitive. Limited to detection of known and characterized plasmid lineages used for primer design. Utility will increase as number of available plasmid sequences grows. Much scope for combination with other strategies for plasmid isolation to facilitate subsequent analysis of plasmid distribution and abundance.

| Plasmid isolation strategy | Advantages | Disadvantages | Potential augmentation/future utility |
|----------------------------|------------|---------------|--------------------------------------|
| **Endogenous isolation**   | - Original bacterial host is known | - Requires host cultivation restricting utility for study of natural communities | - As new organisms are isolated and culture requirements defined, endogenous isolation will continue to be of utility |
|                            | - May be used for all cultivatable bacteria | - Reliance on plasmid-encoded traits if surrogate host species required for plasmid characterization | | |
|                            | - Applicable to all plasmid types | | Compatible with large-scale whole genome sequencing projects |
| **Exogenous isolation**    | - Culture-independent | - Rely on plasmid-encoded traits for plasmid transfer, selection, and maintenance in surrogate host. | - Scope for retrieval of plasmid host range information using RING-FISH, or bioinformatic approaches |
|                            | - Selective isolation of self-transmissible or mobilizable elements | - Original bacterial host unknown | |
|                            | - Potentially capable of isolating all plasmid types (circular and linear), and sizes. | - Range of plasmids isolated dependant on mating conditions used and dictated by numerous "unknown" environmental variables influencing host | |
|                            | - Can isolate plasmids irrespective of abundance in community | | |
| **PCR-Based detection**    | - Culture-independent | - Original bacterial host unknown | - Scope for retrieval of plasmid host range information using RING-FISH, or bioinformatic approaches |
|                            | - High-throughput | - Complete characterization of plasmid detected generally impossible. | |
|                            | - Sensitive | - Limited to detection of known and characterized plasmid lineages used for primer design. | |
|                            | - Scope for accurate quantification of plasmids | | |
| **TRACA**                  | - Culture-independent | - Original bacterial host unknown | - Utility will increase as number of available plasmid sequences grows. |
|                            | - Suitable for development of high-throughput strategies | - Transposon may inactivate genes of interest, impeding phenotypic characterization | |
|                            | - Can isolate plasmids irrespective of abundance in a community | - Currently available Tn elements and surrogate host may limit range of plasmids isolated. | |
|                            | - Fully independent of plasmid-encoded traits | - Linear plasmids not captured | |
|                            | - Sequence-based characterization of plasmids facilitated by known Tn sequence in plasmids | - Transformation step may introduce size bias | |
|                            | - Potentially applicable to all circular plasmids and bacterial communities | Plasmids belonging to the same incompatibility group as Tn origin may not be captured due to stability issues in surrogate host. | |
|                            | - May permit capture of MGE other than plasmids when present as circular DNA molecules | | |
| **Standard Metagenomic libraries (BAC/Fosmid)** | - Culture-independent | - Original bacterial host unknown | - Scope for retrieval of plasmid host range information using RING-FISH, or bioinformatic approaches |
|                            | - Suitable for development of high-throughput strategies | - Likely bias toward numerically dominant plasmids | |
|                            | - Initial capture independent of plasmid-encoded traits | - Screening relies on plasmid-encoded traits expressed in surrogate host species | |
|                            | - Sequence-based characterization facilitated | - Not specifically designed for plasmid capture, and non-plasmid sequences dominate libraries. | |
|                            | | - Generally only incomplete, partial plasmids identified | |
|                            | | - General compatibility of library construction methods with plasmid capture unknown. | |
|                            | | Plasmids belonging to the same incompatibility group as vector (BAC/Fosmid) may not be represented due to instability of clones in surrogate host | |
|                            | | Plasmids belonging to the same incompatibility group as vector may not be captured due to stability issues in surrogate host. | |
| **Table 2. Relative merits of currently available plasmid isolation strategies for investigation of whole communities (modified from references 31 and 58)** |

This approach severely compromises its utility when applied to study the plasmid populations in complex bacterial ecosystems, such as the gut microbiota.\(^{31,33,58}\) In these cases, the high proportion of uncultivated species comprising such ecosystems greatly restricts the range of plasmids that may be accessed with this approach, rendering it of limited value in this context.\(^{31,33}\)

A variation of this approach that may be employed to improve the range of plasmids captured is the direct isolation of plasmid DNA from the mixed microbial population without any prior cultivation, and their subsequent transformation into a surrogate host species for characterization.\(^{53}\) However, this negates a key advantage of the endogenous approach, as information regarding the original host species is lost, and introduces an additional series of potential biases by relying on plasmid-encoded traits, such as the ability to replicate in the surrogate host and the presence of selectable markers, which collectively maintain the restricted

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utility of this approach for studying the plasmid pool in complex microbial ecosystems.33

Exogenous isolation of plasmids. Due to the inherent limitations of endogenous approaches, a range of culture-independent plasmid isolation methods have been developed, which permit access to a greater range of plasmids resident in bacterial communities (Fig. 1; see refs. 33, 58, 188, 189 and 192). The best established of these methods are the exogenous isolation approaches, which rely on the natural ability of plasmids to transfer between species.189 In this approach, plasmids are acquired by utilizing a selectable surrogate host species in bi-parental or tri-parental mating with the donor population under study, during which plasmids resident in the donor species/community may be transferred via conjugation into the selectable

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**Figure 1.** Overview of culture-independent strategies to access plasmids resident in the human gut mobile metagenome.
Subsequently, cells are transferred to media selective for the surrogate recipient strain, as well as plasmid-encoded traits, in order to specifically recover recipient strains that have acquired plasmids. Bi-parental matings can be used to recover self-transmissible conjugal plasmids, while tri-parental matings in which additional donor species carrying a mobilizing helper plasmid are employed, can be used to recover plasmids that are mobilizable but not capable of autonomous transfer.

This exogenous approach permits access to plasmids from the total community regardless of ability to cultivate host species, and has successfully been applied to study a wide range of bacterial ecosystems, including the human gut microbiota. The culture-independent nature of this method is a significant advantage over endogenous approaches, and the acquisition of conjugal or mobilizable plasmids is also of much benefit in identifying elements capable of transfer between host species and important in HGT events within a particular community. However, as with modifications to the endogenous approach described above, information regarding the original host species of plasmids is lost and both methods rely heavily on plasmid-encoded traits such as the presence of selectable markers, or ability to replicate in surrogate host species, which again restricts the range of plasmids that may be isolated. For example, in this approach recipient cells harboring captured plasmids must be selected for based on the ability of plasmids to confer a selectable phenotype on host cells, most typically resistance to antimicrobial agents such as heavy metals, antibiotics, and biocides is used. While this selectivity may be an advantage in some studies, such as those concerned with specifically understanding the plasmid-mediated spread of antibiotic resistance genes, plasmids not encoding such traits or not expressing these in the surrogate host will not be isolated, limiting the utility of this method for studying the mobile metagenome in general, and its role in fundamental microbial ecology and community evolution.

A variety of other factors also influence the range of plasmids that may be captured using this method, including the mating conditions used. The duration, temperature, and use of solid or liquid media can affect the range of plasmid types that are acquired, and for some plasmids the physiological conditions of the host cell, and the presence of environmental cues can influence the initiation of transfer events. Since many organisms in complex communities such as the gut microbiota remain uncultured and almost completely uncharacterized, it seems inevitable that the mating conditions used will be sub-optimal or inhibitory for the transfer of many plasmids, further restricting the fraction of the mobile metagenome that may be sampled and studied with this method.

Detection of plasmids in standard metagenomic libraries. More recently a range of metagenomic approaches which permit culture-independent access to members of the gut microbiota have been established, and applied to the study of microbial ecosystems, including the mammalian gut microbiota. These include strategies that permit access to plasmids and other MGE either through the construction and screening of metagenomic libraries or via the specialized culture-independent TRACA. Recently, Kazimierczak et al. (2009) described the isolation of plasmids or plasmid fragments, from standard metagenomic libraries of the organic pig gut microbiota, including those with the ability to replicate autonomously when liberated from the BAC vector and reconstructed by self-ligation.

While this method successfully identified novel plasmids, drawbacks inherent in exogenous methods remain, including reliance on plasmid-encoded selectable traits, and replication in surrogate hosts for demonstration of autonomous replication. Furthermore, this approach is not designed to specifically capture plasmids, but is focused on the acquisition of any metagenomic DNA fragments encoding genes that confer a phenotype of interest on surrogate host cells, in this case tetracycline resistance. As such, sequences captured are not limited to those originating from complete or partial plasmid fragments that have been cloned, and at present this method is not suitable for the specific exploration of the plasmid population associated with the human gut mobile metagenome. Nevertheless, this approach represents a useful strategy for investigating the potential mobility of genes involved in traits of interest within a bacterial community, and is clearly capable of isolating novel, autonomously replicating plasmids.

Transposon aided capture (TRACA) of plasmids. In contrast to the use of standard metagenomic libraries, the TRACA system has been specifically designed to capture plasmids associated with the mobile metagenome of a microbial community. This system overcomes some of the key limitations of other plasmid isolation systems, and offers several major advantages including an independence from plasmid-encoded traits for plasmid isolation. In the TRACA system, plasmids are captured directly from metagenomic DNA extracts, treated with plasmid-safe DNase, by supplying all plasmids with a defined selectable marker and an origin of replication suitable for the chosen surrogate host species (Fig. 1). This is accomplished by delivering the required genes to plasmids extracted from community members via a transposon, in an in vitro reaction. Following in vitro transposon tagging, plasmids are transformed into surrogate host cells and recovered on media selective for the transposable element. In this way, the transposon effectively retrofits all plasmids in the population with a known selectable marker and suitable origin of replication, permitting subsequent selection and stable maintenance in the surrogate host species independently of plasmid-encoded traits. This greatly enhances the range of plasmids that may be captured and application of TRACA to study plasmids resident in the human gut mobile metagenome has demonstrated its ability to isolate novel plasmids devoid of any conventional selectable markers.

Despite the major advantages offered by the TRACA system, this method does not address all drawbacks of other culture-independent systems, and may also be subject to an additional limitation unique to this approach. At present all plasmids isolated with the TRACA system have been in the smaller size range (~14 kb or smaller). As this system is yet to be exploited on a large scale and across a wide range of bacterial ecosystems it remains to be seen if this represents a true size bias of the
TRACA system, or is a result of a predominance of smaller plasmids in the gut ecosystem.\textsuperscript{31,33} While there is currently no means to provide a definitive survey of plasmid size in a given bacterial ecosystem, there is evidence that physical features of plasmids including size, are responsive to environmental and ecological factors in the same way as bacterial chromosomes.\textsuperscript{52}

Plasmids appear to follow the same trend as chromosomes, of decreasing size and gene content as complexity and stability of the external environment reduces and increases, respectively.\textsuperscript{52} Surveys of plasmid size in relation to host habitat have indicated that fewer large plasmids (\(>100\) kb) are associated with microbial species colonizing higher host organisms (animals and plants), in contrast to terrestrial and aquatic environments.\textsuperscript{52} It was proposed that this may be due to selection for large plasmids in highly complex and heterogeneous environments, such as soil, which encode a wide range of accessory traits allowing the host to adapt to multiple and diverse ecological niches and environmental stresses encountered.\textsuperscript{52} However, there may also be mechanisms or selective pressures extant in certain microbial communities which actively limit plasmid size, but at present any such processes remain obscure. As such, there exists the potential for the gut microbiota to be populated by predominantly smaller plasmids, but such hypotheses are not presently supported by any firm evidence and larger plasmids clearly do infect important members of this community and form part of the gut mobile metagenome (exemplified by the lactobacilli and enterococci megaplasmids\textsuperscript{40-42}). In general, elucidation of the average size and dominant plasmid types of elements resident in the human gut is unknown and awaits the implementation of an in-depth census of the gut-associated plasmid pool.

Overall, it is most likely that both the composition of the natural plasmid population and features of the TRACA methodology will contribute to the characteristics of captured plasmids, and the transformation step in the TRACA system represents a key point at which size bias may be introduced, since smaller elements are transformed with much greater efficiency than larger plasmids.\textsuperscript{31,33} In this regard, size fractionation and amplification of plasmid DNA prior to transposon tagging and transformation may permit larger plasmids to be acquired more readily with this system. In addition, the use of surrogate host species other than \textit{E. coli}, and transposons with a wider range of replication origins should also increase the plasmid range that can be acquired, further enhancing this method.

As with exogenous isolation and the use of standard metagenomic libraries, plasmids isolated by TRACA are also divorced from the original host species and this phylogenetic host-range information is lost.\textsuperscript{31,33} Although not required for subsequent characterization of plasmid-encoded functions, knowledge of the original host range of such plasmids is highly desirable and would greatly facilitate our understanding of the role of the gut mobile metagenome in dissemination and distribution of key community functions.\textsuperscript{30,32} There is, however, significant scope to compensate for loss of host range data in the TRACA system, as well as other culture-independent plasmid capture approaches, by implementing downstream plasmid characterization aimed at retrieving such information.

Retrieval of host-range data following culture-independent plasmid isolation. Several strategies currently exist which theoretically permit the retrieval of host range data following the culture-independent isolation of plasmids; a summary of how these may be integrated into existing culture-independent workflows is presented in Table 2. These include the utilization of fluorescent in situ hybridization (FISH) coupled with fluorescence associated cell sorting (FACS), in which plasmid specific probes may be used in conjunction with FACS to retrospectively acquire intact bacterial cells harboring plasmids of interest.\textsuperscript{194} Recovered cell populations may then be analyzed to determine phylogenetic origin using 16S rRNA gene sequences.\textsuperscript{31,195}

Alternatively a number of purely bioinformatic approaches to determine the phylogenetic origin or affiliation of DNA fragments have been developed, including several specifically designed to recover plasmid host range data.\textsuperscript{196,197} In particular, differences in the relative frequencies of di- or tri-nucleotide sequences in plasmids and chromosomes of bacteria have been used to determine evolutionarily distant host ranges of a variety of plasmids.\textsuperscript{196,200} The absolute average relative abundance of di-nucleotide repeats (or delta-distance) has previously been exploited to identify recently acquired or “foreign” genes in bacterial genomes based on differences in this genomic signature, which remains relatively constant throughout the genome but is noticeably altered in recently acquired DNA.\textsuperscript{197-200}

Since plasmids and other MGE have been demonstrated to acquire the genomic signatures of their long-term hosts over time, and these genomic signatures are discriminatory in terms of bacterial species relatedness,\textsuperscript{197,198,200} this concept has also been applied to identify host-plasmid associations based on similarities in genomic signatures.\textsuperscript{197,198,200} The Mahalanobis distance measurement has also been introduced in order to compensate for variance and covariance effects of di- or tri-nucleotide repeats throughout the genome, and shown to be useful for exploring the long-term hosts of plasmids.\textsuperscript{197}

As more complete bacterial genome sequences become available, and in light of the growing number of genome sequences from various human microbiomes including the gut microbiota, the use of powerful metagenomic based methods for plasmid isolation, combined with bioinformatic approaches to recover information on long-term bacterial hosts in a community, is likely to provide much insight into the gut mobile metagenome and its role in community development. The use of the TRACA system in combination with a subsequent in silico metagenomic analysis of plasmid-encoded functions has already been implemented and revealed not only the potential broad geographic distribution of some plasmids or plasmid families in the gut microbiomes of individuals from across the globe, but also highlighted the unexpected enrichment of some plasmid-encoded functions in this community.\textsuperscript{52}

Direct plasmid detection by PCR. Finally, there also exists the option for the direct detection of plasmids in metagenomic DNA extracts through a PCR-based approach, using primers specific to conserved backbone regions of certain plasmid families.\textsuperscript{56,58} A range of primer sets have been developed to distinguish between plasmids of various incompatibility groups, and these may also be
exploited to determine if plasmids of a particular group are present in a microbial population.\textsuperscript{56,58,192} However, such an approach is extremely limited, and not only is the subsequent characterization of plasmids virtually impossible, the plasmids that may be detected are limited to those for which well-characterized relatives already exist which can be used for primer design.\textsuperscript{31}

Despite this, such approaches will likely be of value in augmenting other culture-independent metagenomic strategies, facilitating elucidation of plasmid host ranges, geographic distribution, and inter-individual variation between gut microbiomes (Table 2). PCR-based approaches are also well suited to determine the general prevalence and overall abundance of particular plasmids of interest within a community through quantitative PCR, and would permit fluctuations in the population size of plasmids of interest isolated by methods such as TRACA, to be monitored over time within an individual or in response to changes in environmental parameters.

**Summary and Future Perspectives: What Can the Mobile Metagenome Tell Us?**

Although MGE have been extensively studied from the perspective of gene flow among bacteria, the evolution of bacterial species, and the spread of antibiotic resistance genes, few studies have considered the role of MGE at the community level and the contribution of the mobile metagenome to the development of community functions. In particular, the study of the mobile metagenome in microbial ecosystems that have co-evolved with higher host organisms, such as the gut microbiota, are likely to reveal important insights into the development of this community, its functional output, and interaction with the metazoan host.\textsuperscript{30–33,89}

There is increasing evidence that the gut mobile metagenome reflects the co-evolution of host and microbe (Table 1),\textsuperscript{20,30,32,48–50,201} and as such contains MGE and encodes functions important to bacteria inhabiting this environment. Recently, the integration of the mobile metagenome into the hologenome model of evolution has been proposed and described.\textsuperscript{50} The hologenome theory builds on the increasing appreciation that humans and other complex metazoans are in essence amalgams of both prokaryotic and eukaryotic cells, resulting from the co-evolution of host and microbe,\textsuperscript{4,5,8,30} and proposes that the basic unit of selection in evolution consists of the total genetic content of the eukaryotic host plus that of all associated microbial partners.\textsuperscript{4,5} However, it should be noted that alternative and popular evolutionary theories place the emphasis on selection at the level of the gene, but this view is not necessarily incompatible with the hologenome theory (in which success of the holobiont would also equate to success of genes constituting the hologenome) and it is likely that selection acts at multiple levels.

One way in which the hologenome arrangement is proposed to greatly benefit the human host is by increasing adaptability to new environmental conditions and exploitation of food sources.\textsuperscript{4,5,30} This stems from the plasticity of prokaryotic genomes, which by virtue of their short generation times and ability to acquire new functions through HGT, are infinitely more flexible than the relatively static “human” genome.\textsuperscript{4,5,30} In the proposed integration of the gut mobile metagenome into this model, the genetic information encoded by the prokaryotic complement of metazoans constitutes a secondary accessory genome, and the mobile metagenome a highly flexible tertiary gene pool which provides access to the wider prokaryotic gene pool, and serves to further enhance the adaptability of the holobiont as a whole through HGT.\textsuperscript{50}

Furthermore, MGE have also been proposed to be important in the maintenance of cooperation between bacteria, and therefore the mobile metagenome may illuminate mechanisms through which the community maintains cooperation and social stability, and curtails the ingress of cheaters.\textsuperscript{202,203} Many genes involved in the production of beneficial traits that may be exploited by a population as a whole (public goods) such as secreted enzymes, are encoded by MGE.\textsuperscript{202,203} There is growing evidence that genes required for the production of such public goods may be stratified between MGE and core bacterial genomes, with the mobile metagenome encoding a greater proportion of activities involved in the production of these public goods.\textsuperscript{202,203}

The maintenance of such genes on MGE, rather than their fixation into core genomes of relevant bacterial species, may be due to a number of selective pressures. This includes temporal and spatial variation in selective pressure for the traits encoded, but also the possibility that mobility of these genes permits the population to rehabilitate individuals that loose genes required for generation of public goods, and subsequently exploit a public good without contributing to its production (referred to as cheaters).\textsuperscript{8,203} These cheaters generally exhibit a fitness advantage over other members of the population and in the case of the human gut microbiota, it is predicted that the emergence of cheaters will destabilize community functions adversely affecting the human host, and so have deleterious effects on the community and the holobiont as a whole.\textsuperscript{8} It has been proposed that the infection or re-infection of these cheaters with MGE encoding genes required for production of the public good in question, serves as a mechanism by which their emergence in a population may be controlled and sociality maintained.\textsuperscript{202,203}

The role of the gut mobile metagenome in regulating this aspect of community function has not yet been subject to significant study, but the contribution of this flexible gene pool to policing or rehabilitation of cheaters, and maintenance of cooperation in the gut microbiota presents an intriguing possibility that should be investigated in more detail. Of particular interest in this context is the potential prevalence of RelBE TA modules in gut-associated plasmids, which enhance the stability of MGE in a bacterial host through a post segregation killing mechanism (in which loss of MGE encoding the TA module results in growth arrest or cell death), and so could ensure contribution to the production of a public good is maintained by enforcing carriage of MGE encoding the necessary genetic information.\textsuperscript{32,202} Recently, Noguiera et al. (2009) highlighted not only the association of genes in the E. coli secretome (producing public goods) with MGE, but also the association of these with “mafia strategy” addictive systems such as TA modules, which were proposed to promote their maintenance.\textsuperscript{202}
Overall, the gut mobile metagenome constitutes a vast and untapped reservoir of novel genetic information, which has the potential to greatly enhance our understanding of this complex community, as well as facilitating the development of strategies to modulate the functions of this ecosystem for the benefit of human health. Therefore, accessing the wealth of novel genetic data that is likely to reside in the human gut mobile metagenome will not only illuminate important, fundamental aspects of community function and development, but will also facilitate the discovery and exploitation of novel MGE or MGE-encoded activities of biotechnological or clinical value. For example, MGE enriched or specific to this ecosystem have already been exploited as novel tools for microbial source tracking to determine the origin of faecal pollution in surface waters.47

The study of host-associated microbial ecosystems such as the gut microbiota, and in particular the community level analysis of associated mobile metagenomes, constitutes a new frontier in microbiology, the opening of which will lead great intellectual reward. Although significant technological development is still required to facilitate the full exploration of this new territory, strategies that permit the first forays into this promised land are now in place, and initial expeditions have been mounted. However, few studies have so far examined the mobile metagenomes of such communities in detail, and future projects aimed at characterizing host associated microbiomes should incorporate the study of associated mobile metagenomes as a key component.

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