Barriers to Horizontal Gene Transfer: Fuzzy and Evolvable Boundaries

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Abstract

The existence of numerous types of barriers to horizontal gene transfer (HGT) is well documented. Nevertheless, no barrier is impervious, and all kinds of genes can occasionally find their way into organisms different from the ones in which they evolved. This by no means implies a free flow of genes across taxa, but, rather, the existence of complex networks of loopholes across barriers that could potentially connect all kinds of organisms through gene transfer. Loopholes may be provided by way of a small fraction of the individuals in an otherwise inaccessible population and, if exogenous genes are incorporated into the chromosome, homology-assisted recombination processes may further spread them across wild type individuals. HGT networks should be very fluid, as they depend heavily on fortuitous events and transient circumstances, such as the presence of mobile genetic elements (MGEs) in a potential donor that may extend the transfer network in particular directions. Moreover, HGT networks should be highly evolvable, as a result of (1) the multiplicity of processes with the potential to modulate, modify or alleviate the different barriers to transfer, (2) the constantly changing selective pressures operating on them and (3) the enormous plasticity of MGEs. Finally, HGT networks should be gene-specific, as different genes should have an unequal likelihood of passing through or being incorporated into new genomes.

Introduction

The controversy surrounding HGT is often polarized into views that attempt to summarize the overall impact of this phenomenon on prokaryotic evolution, particularly regarding the existence of prokaryotic species and of organismal phylogenies (Bapteste and Boucher, 2009; Doolittle and Zhaxybayeva, 2009; Puigbo *et al.*, 2009; Riley and Lizotte-Waniewski, 2009). HGT, however, encompasses a multiplicity of processes, which operate through different agents and mechanisms, rely on different ecological circumstances and result in a wide range of fitness effects on the recipient organisms. In this chapter, we wish to explore the barriers that limit HGT among organisms and the circumstances and manners in which they may be circumvented, following the rationale that taking into account the conditions in which HGT operates should contribute to the development of more specific lines of research on the detailed dynamics of HGT processes and their repercussions on the evolution of prokaryotes.

Ecological opportunity

HGT is a widespread phenomenon for many organisms. Cases have been documented involving all possible pair-wise combinations and directions of transfer in the three domains of life plus viruses (Nelson *et al.*, 1999; Boucher *et al.*, 2001; Beiko *et al.*, 2005; Choi and Kim, 2007; Keeling and Palmer, 2008; La Scola *et al.*, 2008; Hecht *et al.*, 2010; Dunning Hotopp, 2011). This may conduce to thinking that HGT is frequent at all biologically relevant levels and time-scales. Given the number of individual organisms inhabiting our planet and the time elapsed since cellular life became established on Earth, a high frequency of HGT would certainly lead to erasing most, if not all, signs of vertical inheritance. Several authors support this conclusion (Hilario and Gogarten, 1993; Glansdorff *et al.*, 2008; Koonin and Wolf, 2009) but, by large, HGT has not completely blurred the phylogenetic signal resulting from vertical transmission of genetic material. In consequence, we need to understand how different factors and processes enable or limit horizontal gene transfer among organisms.

The first step for an HGT event is the contact of the foreign genetic material and the recipient organism. We will consider the different ways in which such contacts may happen in a different section and will start here by dealing with their ecological context.

Different environments determine which assemblages can donate/receive genes from other members thus creating special groups or exchange communities. Also, the type of genes laterally transferred among members of such communities might be different because of the disparate selective advantages resulting from the transfer of specific genes in different environments. Both the size and composition of the gene pool available to be incorporated into the genome of an organism by HGT, also known as its 'supergenome' (Norman *et al.*, 2009), are dependent on the environment it inhabits. The environment not only determines the selective framework but also the set of genetic innovations accessible to cope with it, and HGT is the most common mechanism for their acquisition (Pallen and Wren, 2007). For instance, according to Pál *et al.* (2005), most changes to the metabolic network of *Escherichia coli* in the past 100 million years have been due to HGT, with little contribution from gene duplication. Furthermore, genes incorporated by HGT are mainly involved in the transport and catalysis of external nutrients, thus driving the adaptations to changing environments.

The preferential exchange of genetic material among species inhabiting the same environments may lead to the emergence of assemblages of species or communities which become bound together not only through adaptation to similar conditions but also through access and sharing of an almost common gene pool. This view has been favoured by some large scale analyses of lateral transfer which have identified preferential routes or 'highways' of gene sharing between taxa with common phylogenetic history and environmental distribution (Beiko *et al.*, 2005), even leading to the proposal of separate, independent 'genetic worlds' (Halary *et al.*, 2010), defining multiple isolated, distinct gene pools.

The phylogenomic analysis of gene content and distribution of clusters of orthologous genes (COGs) among bacteria classified according to lifestyle led Audic *et al.* (2007) to conclude that clustering results from vertical inheritance and lateral gene transfer, which varies in extent and nature of transferred genes among environments. This analysis focused on *Minibacterium massiliensis*, a water-living Betaproteobacteria, and allowed the authors to conclude that bacterial communities living in this habitat exhibited the highest proportion of horizontally transferred genes, especially for three functional categories: C-COG (energy production and conversion), D-COG (cell division and chromosome partitioning) and I-COG (lipid metabolism).

Environmental conditions may have a strong effect on the actual mechanism of HGT and, in consequence, on the likelihoods and frequencies of different processes (Norman *et al.*, 2009). For instance, Charpentier *et al.* (2011) have recently shown that environmental stress conditions affect the state of competence for transformation in *L. pneumophila* by means of changes in the expression of competence-related genes such as *comEA*. As a result, the chances of transformation with foreign DNA may increase in stressing environments.

However, other mechanisms seem to play a more important role than transformation in certain environments. Kenzaka *et al.* (2010) have recently evaluated the rate of gene exchange among bacteria mediated by phages in aquatic environments, estimating values as high as 2×10^{-3} per total direct count when natural bacterial communities were recipients. McDaniel *et al.* (2010) analysed the purple non-sulfur bacterium *Rhodobacter capsulatus,* which inhabits marine environments. They found that gene transfer agents (GTAs), host-encoded virus-like sequences found in the genome of most Rhodobacterales, have contributed several orders of magnitude more often to the incorporation of new genetic material into the *R. capsulatus* genome than transformation and transduction. A large analysis of almost 120,000 homologous DNA families (Halary *et al.*, 2010) has recently concluded that plasmids and other vehicles of gene exchange have been and still are mainly responsible for gene exchange among bacterial chromosomes.

Potential hot spots in the environment for recombination and HGT include biofilms, the rhizosphere, decomposing material, guts of soil animals and the interior of bacterivorous protozoa (Ragan and Beiko, 2009). Hence, it is not surprising that high rates of HGT are found in species commonly found in these habitats, with a high proportion of genes transferred from other members of the same communities. At the other end, obligate intracellular bacteria have been thought to be largely unaffected by HGT owing to their isolation from potential donors (Renesto *et al.*, 2005). Nevertheless, *Buchnera*, obligate endosymbiont bacteria of aphids, still present prophages, plasmids and transposons (Bordenstein and Reznikoff, 2005) and some evidences point towards lateral gene acquisition after starting their obligate lifestyle (Van Ham *et al.*, 2000). Similar observations have been made in other intracellular parasites such as *Rickettsia felis* (Gillespie *et al.*, 2008) or *Chlamydia trachomatis* (DeMars *et al.*, 2007).

However, some evidence indicates that there is not an absolute necessity of sharing the same environment for the lateral transfer of genes between bacterial species. For instance, although generalist species, such as *Pseudomonas aeruginosa*, have large genomes and can survive in a wide range of habitats, it is somewhat surprising that a clinical strain of this species harbours genes for the degradation of secondary metabolites of trees (Mathee *et al.*, 2008). Amoebas can act as a common ground for the exchange of genes among the tens of bacterial species that inhabit, regularly or occasionally, their cytoplasm (Coscolla *et al.*, 2011), and viruses are capable of moving between habitats (Breitbart and Rohwer, 2005). In consequence, the environment plays a major role in the frequency, nature, and mechanism of horizontal gene transfer but exchange communities (Jain *et al.*, 2003) cannot be considered as closed.

Host range of different HGT mechanisms

Numerous bacterial species, belonging to very different phylogenetic and trophic groups, are naturally competent for transformation by free exogenous DNA (Lorenz and Wackernagel,

1994). In addition, an enormous variety of MGEs contributes to the spread of genes across bacterial cells. MGEs can vary widely in terms of the range of organisms that can serve as their hosts and among which they can transfer genetic material. We will explore the taxonomic breadth across which different HGT mechanisms can operate, review some of the factors that contribute to delineate this range, and analyse how interactions among different MGEs in a host cell or bacterial community can affect it.

Transformation

Transformation is the active uptake of exogenous DNA by competent cells followed by genomic integration. Naturally transformable species have been detected in all the major taxonomic groups of Eubacteria as well as in the Archaea (Lorenz and Wackernagel, 1994; Kleter *et al.*, 2005). Unlike genes responsible for conjugation and transfection, which are MGE-encoded, the genes required to render a cell transformable are usually encoded by the bacterial chromosome, and often dispersed across different chromosomal regions, so that transformation could be considered as the genuine prokaryotic sex (Lorenz and Wackernagel, 1994).

For natural transformation to occur, DNA has to be released from donor cells and dispersed or maintained in the environment until being encountered by potential recipient cells. Numerous studies have demonstrated that extracellular DNA of multiple sources can be present at high concentrations in diverse prokaryotic habitats and persist for considerable time (Nielsen *et al.*, 2007). On the other hand, recipients need to develop competency via the highly regulated expression of specific proteins and complexes that may include sophisticated DNA uptake systems. Generally, competent bacteria can translocate chromosomal DNA fragments, plasmids or phage DNA across their membrane(s) (Thomas and Nielsen, 2005; Brigulla and Wackernagel, 2010). The actual translocation process varies among taxa, but usually involves the transfer of single DNA strands (Chen *et al.*, 2005). Natural transformation machineries have been studied in a variety of bacteria, including *Bacillus subtilis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria gonorrhoeae* and *Helicobacter pylori*. Although not exclusively, most of these DNA uptake machineries are related to type IV pili and type II secretion systems (Lorenz and Wackernagel, 1994; Averhoff and Friedrich, 2003; Chen and Dubnau, 2003). Importantly, although most uptake systems can transport DNA from diverse sources, for some of them the species of origin of the DNA can strongly affect the likelihood of its uptake. In *H. pylori*, which uptakes DNA by means of a reverse type IV secretion system (Smeets and Kusters, 2002), intraspecific DNA is preferentially uptaken even in the presence of 1000-fold excess of DNA molecules from other sources; this result was obtained with both genomic DNA and PCR products, indicating that *H. pylori* and foreign DNA are not differentiated by methylation patterns but at the level of primary DNA sequence (Levine *et al.*, 2007). Specific DNA uptake sequences have not yet been identified in this species, but are well characterized in other naturally transformable bacteria such as *Haemophilus* and *Neisseria* (Saunders *et al.*, 1999). These organisms preferentially take up DNA from their own clades, which they recognize by 9- or 10-nucleotide-long sequences that are overrepresented in their genomes (Thomas and Nielsen, 2005). In contrast, the Gram-positive *B. subtilis* and *S. pneumoniae* and the Gram-negative *Acinetobacter* sp. and *Pseudomonas stutzeri* do not recognize specific sequences and can similarly uptake homologous and heterologous DNA (Averhoff and Friedrich, 2003).

Therefore, selectivity in the transformation process can exist at the level of exogenous DNA recognition, but the degree to which it occurs is extremely variable across bacterial species.

Interestingly, recent work with *Streptococcus* has revealed that competent cells can trigger DNA release from non-competent cells belonging to the same or closely related species, via what seems to be an active mechanism to secure a homologous DNA pool for uptake and recombination (Johnsborg *et al.*, 2008). This phenomenon involves a fratricide mechanism that is co-regulated with natural transformation. Competence for natural transformation is a transient strictly regulated physiological state in *Streptococcus* and many other bacteria and can be induced by a variety of stressors. In the *streptococci*, it can be induced by high concentrations of a secreted pheromone termed the competence-stimulating peptide (CSP), for which there exists a variety of types specific to limited subgroups of strains termed pherogroups (Havarstein *et al.*, 1995). When pherogroup members sense a critical concentration of their specific CSP, they develop the competent state and release lysins to which they are immune but that kill neighbouring cells belonging to other pherogroups. This competence-induced cell lysis has been experimentally shown to increase the efficiency of lateral gene transfer from conspecific streptococci by 1000-fold, and from closely related species by 40-fold. Similar systems of co-induced competence and bacteriocin production by CSPs have been found in other streptococci and in *B. subtilis* (Kreth *et al.*, 2005). It is clear that such systems have the potential to give rise to arms races that have likely contributed to generate the great variety of CSP molecules and cognate receptors that exist in nature (Johnsborg *et al.*, 2008).

Mechanisms that will favour the intracellular survival, genomic integration and expression of transformed DNA from closely related organisms over more divergent ones will operate once DNA has been introduced into the recipient cell. As the different factors that contribute to this selectivity will also operate for the other mechanisms of HGT, they will be discussed in a more general context in the section 'Factors operating across HGT mechanisms'.

Transduction

Because of their prevalence, phages are likely to play an important role in gene transfer in the environment. Although it used to be thought that phages only rarely crossed species boundaries, broad-host-range (BHR) generalized transducing phages that can transfer genes between species and genera have recently been discovered at high frequency in different natural environments (Nzula *et al.*, 2000; Langley *et al.*, 2003; Sullivan *et al.*, 2003). In some cases, transduction can even occur among bacteria belonging to different classes. For instance, phage SN-T can infect species within the Gammaproteobacteria, Alphaproteobacteria and Betaproteobacteria (Jensen *et al.*, 1998; Beumer and Robinson, 2005). Moreover, the range of organisms into which a phage can inject its DNA can be wider than the actual infective host range (Jensen *et al.*, 1998; Holmfeldt *et al.*, 2007).

Bacteria possess diverse types of resistance mechanisms to avoid infection by phage. Interestingly, some of these mechanisms are actually encoded themselves by prophages or plasmids. Although they serve mainly to ensure bacterial survival, resistance mechanisms are also relevant in defining phage host range and therefore the potential for transduction among different organisms. Phages, in turn, employ numerous resistance countering, and therefore host-range expanding, adaptations (Hyman and Abedon, 2010). As a result,

complex arms races can develop involving the phage, the host bacterium and the different MGEs that it might contain. In addition, bacteria are often found in biofilms, which can diminish their susceptibility to phage infection.

As for transformation, the first level of resistance to phage can occur at the bacterial cell surface, at the level of phage attachment to cognate receptor molecules. Interaction between phage and bacterium can be reduced by loss, down-regulation or structural alteration of phage receptor molecules. Phage receptors, though, often have important roles in cell biology and tampering with them may entail substantial fitness costs. Nevertheless, mutations to adsorption resistance through these processes are common (Hyman and Abedon, 2010), speaking to the high selection pressure exerted by phages. Some bacteria have evolved very specific mechanisms to turn on and off the expression of a given receptor. In *Campylobacter jejuni*, invertible genome segments bound by Mu-like prophage segments control the production of a bacteriophage receptor, flagellin. Again, this system is costly to the cell, as, in the flagellin-off stage, flagella loss reduces pathogenicity and the Mu-like prophage genome is restored and activated, leading to production of infectious phage (Scott *et al.*, 2007). Other prophages are known to interfere with the display of their own cell-surface receptors (Perry *et al.*, 2009).

Phages attach to their host receptors via specific molecules, known as antireceptors. Possibly in order to counteract the host capacity to alter receptor molecules, phage antireceptors are often very flexible and able to recognize several bacterial surface molecules (Goldberg and Murphy, 1983; Montag *et al.*, 1990; Hyman and Abedon, 2010). In some cases, phages encode multiple antireceptor genes for different receptors, displaying more than one antireceptor in the same virion (Scholl *et al.*, 2001; Leiman *et al.*, 2007) or utilizing site-specific genome inversion mechanisms to switch between antireceptors (Hyman and Abedon, 2010). Phages can further respond to a receptor mutation in their hosts by changing their antireceptors so that they recognize the altered receptor or a different one, or by becoming less stringent in terms of receptor recognition. Remarkably, diversity-generating systems specifically targeting antireceptor genes have recently been discovered, such as the reverse-transcriptase-mediated, site-specific antireceptor gene mutation system of *Bordetella* phages BPP-1, BMP-1, and BIP-1 (Liu *et al.*, 2002; Medhekar and Miller, 2007). Logically, antireceptor-modifying mutations may indirectly result in shifted or expanded phage host ranges. Relaxed host specificity can also result from conformational changes induced by modification of phage proteins other than the antireceptor molecule (Hyman and Abedon, 2010).

Rather than eliminating, downregulating or modifying phage receptor molecules, bacteria can also conceal them by means of physical barriers, such as capsules and other extracellular structures. However, such barriers can also be circumvented by the phage, via enzymes that degrade their constituent polymers. Typically, these enzymes are active against only a narrow range of barrier types (Sutherland *et al.*, 2004; Scholl and Merril, 2005) and extracellular polymers should block infections by only certain phages. Therefore, the broad chemical diversity of extracellular barriers among bacterial strains could be the product of a phage-mediated frequency-dependent selection (Abedon, 2009) where rare barrier types would be less subject to barrier-circumventing phages (van der Ley *et al.*, 1986; Scholl and Merril, 2005). Furthermore, while extracellular polymers reduce infection susceptibility to some phages, they can increase susceptibility to other phages that may recognize them as receptor molecules (Pelkonen *et al.*, 1992; Hanfling *et al.*, 1996; Weinbauer,

2004). Consequently, arms races could develop involving a variety of competing phage and bacterial strains.

After adsorption, different mechanisms can operate to avoid the delivery of the phage genome to the cytoplasm. These mechanisms can be encoded by plasmids and prophages or by the bacterial chromosome (Hyman and Abedon, 2010). If the phage genome reaches the cytoplasm, several other processes can still interfere with its integrity or its capacity to integrate into the bacterial genome. These processes are addressed below in the section 'Factors operating across HGT mechanisms'. Finally, some bacteria may respond to phage infection by employing measures that will cause the infected cell to die, presumably to decrease the probability of infection of other cells in the population. A widespread mechanism of this sort involves nucleases that, when activated, cleave the anticodon loop of a specific tRNA, blocking protein synthesis (Hyman and Abedon, 2010). Some phages, such as phage T4, however, are able to repair these cuts, while others have evolved alternative means of resistance (Amitsur *et al.*, 1987; Labrie and Moineau, 2007; Haaber *et al.*, 2009).

Conjugation

Amongst the bacterial gene exchange mechanisms, transformation, transduction and conjugation, the latter has the broadest host range of transfer (Dreiseikelmann, 1994; Droge *et al.*, 1998). Conjugation is widespread among Gram-negative and Gram-positive bacteria and occurs also in Crenarchaeota (Stedman *et al.*, 2000). It is mediated by plasmids or by integrative conjugative elements (ICEs). A conjugative plasmid recombined into the chromosome can also initiate conjugal transfer of chromosomal DNA. Conjugative elements are able to induce the formation of mating pairs between a donor and a recipient cell. Conjugative plasmids and ICEs of Gram-negative bacteria encode filamentous structures called *pili* that aid in mating-pair formation (Beaber *et al.*, 2002; Burrus *et al.*, 2002), whereas elements that are restricted to Gram-positive bacteria produce surface-presented protein factors that induce cell aggregation (Clewell, 1993; Godon *et al.*, 1994; Burrus *et al.*, 2002). In the best-studied conjugation system, the one mediated by plasmids of Gram-negative bacteria, the donor cell produces a mating pair formation (Mpf) system that enables intimate physical contacts with a recipient cell through a membrane-spanning protein complex and a surface-exposed sex pilus. Additionally, a coupling protein (CP) interacts with the DNA substrate and couples it to the secretion pore formed by the Mpf system. Mpf/CP conjugation systems belong to the family of type IV secretion systems, which also includes DNA uptake and release systems, as well as effector protein translocation systems (Schroder and Lanka, 2005). The translocated DNA is a single strand that starts from an origin of transfer (oriT) and enters the recipient cell directed by a relaxase protein covalently joined to the 5′ DNA end (Lee and Grossman, 2007). Much less is known about how DNA traverses the recipient cell membranes. Pérez-Mendoza and de la Cruz (2009) performed a systematic analysis of the contribution of each gene in an *Escherichia coli* recipient to the conjugal transfer of IncW plasmid R388. Their results indicated that no non-essential recipient genes are required or affect conjugative DNA processing or transport in a significant manner, although mutations in lipopolysaccharide synthesis pathways can impair mating pair formation, particularly in liquid matings. This screen did not reveal any molecule that might serve as a 'conjugation receptor', analogous to the receptor molecules of transformation and transfection. Therefore, conjugal DNA transfer appears to be a process that is basically carried out by the donor cell and where

the recipient has little say (Perez-Mendoza and de la Cruz, 2009). A 'shoot and pump' conjugation model (Llosa *et al.*, 2002) has been proposed where the sheer push force imparted by the type IV secretion system on the DNA causes the transport machinery to act as a syringe or a drilling machine for injecting the DNA into the recipient cell.

After DNA entry into the recipient, the requirements of plasmids and ICEs for replication and maintenance differ, and we will discuss them, and their effects on host range, separately in the next two sections.

Plasmids

Once inside a recipient cell, the transferred plasmid DNA has to be reconstituted into a replication-proficient form. In many cases, this process must involve, at least, recircularization of the transferred DNA strand and synthesis of a complementary, lagging strand. In the analysis of recipient gene contribution to conjugal transfer performed by Pérez-Mendoza and de la Cruz (2009), no specific recipient enzymes appeared to be required for the reconstitution of a replication-proficient plasmid. The IncW R388 plasmid used in their experiments encodes a relaxase that is transported to recipient cells during bacterial conjugation and is likely responsible for DNA strand recircularization (Garcillan-Barcia *et al.*, 2007). However, R388 does not encode a DNA primase for lagging strand synthesis, and it probably relies on the host RNA polymerase, an enzyme essential for cell survival, as occurs for plasmids that employ a rolling-circle mode of replication (Kramer *et al.*, 1997). In other systems, the primase is plasmid-encoded and its transport to the recipient cell is essential for conjugation in some heterologous matings (Merryweather *et al.*, 1986). For plasmids that rely on host relaxases and primases, the ability of these enzymes to be functional on plasmid DNA will determine if a functional plasmid can be reconstituted after transfer and contribute to define whether a given species can be counted within the plasmid's host range.

Probably, the single most important factor in determining plasmid host range is the capacity of sustaining replication in a particular recipient after transfer and reconstitution (Guiney, 1982; del Solar *et al.*, 1996). Plasmids that can be replicated with fewer host-dependent requirements can successfully replicate in more cytoplasms. A critical time point is the initiation of replication, as plasmids that require host proteins for this process are limited to bacteria whose proteins are able to interact productively with the plasmid's replication origin (Caspi *et al.*, 2000). In contrast, BHR plasmids often initiate replication in an autonomous manner. This is the case for the very promiscuous IncQ plasmid RSF1010, which can be transferred and replicate among Proteobacteria, Firmicutes, Actinobacteria and Cyanobacteria, and even reach eukaryotic organisms including yeasts, plants, and animals (Gormley and Davies, 1991; Sode *et al.*, 1992; Nesvera *et al.*, 1994; Rawlings and Tietze, 2001). IncQ plasmids do not encode their own conjugative machinery, but they can be mobilized by a variety of conjugative elements and transmitted by means of different types of type IV secretion systems, which also contributes greatly to their very broad host ranges. They replicate by a strand-displacement mechanism and contribute to their replication by means of three proteins: RepC, which binds specifically to an oriV origin of replication; RepA, a 5′ to 3′ DNA helicase; and RepB, a DNA primase (Scherzinger *et al.*, 1991). Comparative analyses using cell extracts of *E. coli* and *P. aeruginosa* strongly suggest that IncQ plasmids employ the same mechanism of replication in different hosts (Diaz and Staudenbauer, 1982).

Plasmids that do rely on host proteins for initiating replication can nevertheless have very broad host ranges, as long as they exhibit substantial flexibility in terms of their interaction requirements (del Solar *et al.*, 1996; Doran *et al.*, 1999). This is the case of plasmids belonging to the IncP-1 group, which are among the most promiscuous plasmids known so far. IncP-1 plasmids have the ability to transfer between and replicate in nearly all species of the Alpha-, Beta- and Gammaproteobacteria, and, for the IncP-1 plasmid pKJK10, transfer has also been detected between Proteobacteria and Actinobacteria cohabiting the rhizosphere of barley plants (Musovic *et al.*, 2006). IncP-1 plasmids employ a θ replication mode that starts at an oriV replication origin and encode the initiation protein TrfA. The initiation of IncP-1 plasmid replication also requires several host proteins, including DnaA, DnaB and DnaC (Pinkney *et al.*, 1988). DnaA modulates the action of TrfA by binding to DnaA boxes and stabilizing the formation of the TrfA-mediated open replication–initiation complex. Genetic information indicates that, at least in the IncP-1α subgroup, host promiscuity is partially linked to a modular organization of oriV, as this origin contains several DnaA boxes that are differentially bound by DnaA in different hosts (del Solar *et al.*, 1996). Bound DnaA interacts with DnaB helicase to form a DnaABC complex involved in priming. The process of DnaB loading and activation at oriV may also be different in various hosts (Caspi *et al.*, 2000). Moreover, the observation that DnaA boxes are not required for the replication of IncP-1α plasmids in *P. aeruginosa* raises the possibility of an alternative mechanism for the stimulation of open complex formation and DnaB loading at oriV (Doran *et al.*, 1999).

In contraposition to BHR plasmids, which are able to transfer across different phyla and even across the prokaryote/eukaryote barrier, those that have more restricted transfer capacities are termed NHR (narrow host range) plasmids. Some NHR plasmids can transfer across family-level groups of bacterial hosts, whereas others are apparently restricted within a single bacterial genus. However, selection experiments have been successful at enlarging the host-range of genus-specific plasmids via, for instance, mutations in the plasmid gene encoding the initiator of replication protein (Garcia de Viedma *et al.*, 1996). Thus, minor genetic changes in a plasmid replication protein can expand the plasmid host range (del Solar *et al.*, 1996). Nevertheless, other factors may restrict host range enlargement in nature, as phylogenetic analyses have shown that NHR plasmids can have tight, long-term evolutionary associations with specific host genera. This is the case of the RepA_N family of plasmids, which is broadly distributed in Gram-positive bacteria but where each member of the family has a narrow, intrageneric, host range. Congruence between bacterial host phylogenies and those based on the replication-initiator protein RepA indicates that this plasmid family predated the divergence of the Firmicutes and that different plasmids coevolved with specific genera. Other RepA_N plasmid loci, however, have undergone different phylogenetic histories, indicating a modular evolution for this plasmid's genome (Weaver *et al.*, 2009).

The long-term maintenance of a plasmid in a given host necessitates other conditions besides effective replication. For instance, the BHR IncQ plasmids also ensure their host range by virtue of a partitioning system that is active in a number of different hosts (Siddique and Figurski, 2002) and, in some groups of plasmids, such as IncQ2, by means of toxin-antidote systems that contribute to plasmid stability when copy-number is low (Deane and Rawlings, 2004). These mechanisms, added to their capacity of being mobilized by a variety of conjugative elements and to their autonomous initiation of replication, ensure that IncQ plasmids not only have one of the broadest host ranges of all MGEs, but may just be amongst the most successful plasmids in nature in terms of their prevalence in a variety of

environments (Meyer, 2009). Another interesting case is that of IncP-1 plasmids. Although these plasmids can replicate in numerous different hosts, they are not maintained with equal efficiency in each of them. Importantly, plasmid stability does not correlate with the phylogenetic relatedness of the hosts, but rather seems to be strain specific, varying greatly within the same genus or even the same species. This implies that plasmid stability can be affected by rapidly evolving host-encoded factors (De Gelder *et al.*, 2007; Heuer *et al.*, 2007). On the other hand, experimental evolution studies have shown that IncP-1 plasmids are able to adapt to hosts in which they were initially unstable, and thereby expand their long-term host range (De Gelder *et al.*, 2008). In nature, adaptation to new hosts is likely to be aided by recombination among different IncP-1 plasmids, which has been shown to be a prominent feature of their evolution (Norberg *et al.*, 2011). Interestingly, the actual host range of IncP-1 plasmids in bacterial communities can also be affected by the identity of the bacteria *donating* the plasmid and by environmental conditions (De Gelder *et al.*, 2005). Given all of the factors involved in defining their host range, IncP-1 transfer networks, and those of many other plasmids, could be highly non-random in nature and vary greatly among different environments.

Integrative conjugative elements

ICEs are a diverse group of genetic elements that encode the functions required to integrate into their bacterial host chromosome and to transfer between cells by conjugation. Upon induction, ICEs excise from the chromosome by site-specific recombination, transfer the resulting circular form by conjugation and integrate by recombination between a specific site of this circular form and a site in the genome of their host. Although their replication and stability is basically ensured by way of their integration into the host genome, some ICEs have recently been shown to be also able to replicate in an autonomous manner after excision; this step does not seem to be required for mating, but apparently facilitates reinsertion of the element into the donor chromosome by increasing its copy number and maintaining it through several generations (Lee *et al.*, 2011). The ICEs include conjugative transposons and conjugative transposon-like genomic islands, as well as numerous unclassified MGEs. They can be found both in the chromosome and in plasmids and they show enormous variation in their genetic organization, the genes responsible for their insertion and excision and the accessory genes they carry. The degree of target site specificity of ICE insertion reactions also varies greatly, even for elements that use closely related enzymes (Roberts *et al.*, 2008). Some elements can also use specific sites in one host and multiple sites in a different host if the preferred site is not present (Wang *et al.*, 2000), as has been shown for Tn916 in different strains of *Clostridium difficile* (Hussain *et al.*, 2005).

Genome sequence analyses suggest that ICEs are widespread in bacteria. Therefore, like phages and plasmids, they probably contribute importantly to horizontal gene transfer. Also similarly to other MGEs, host range varies widely among different ICEs. Some elements transfer only between co-specific bacterial strains, whereas others transfer between different species, genera or phyla (Clewell *et al.*, 1995; Scott and Churchward, 1995; Burrus *et al.*, 2002). For example, the conjugative transposon Tn916 has a very broad host range and can transfer from *Enterococcus faecalis* to a large number of Firmicutes and to various Proteobacteria (Clewell *et al.*, 1995).

ICEs show a highly modular gene organization and mode of evolution and are able to exchange gene modules among themselves as well as with plasmids, phages and

non-conjugative transposons. Promiscuous module exchange probably contributes significantly to the evolution of both integration and transfer specificity in ICEs and, as the integration and conjugation functions are encoded by different modules, these two properties can probably evolve and affect host range quite independently of each other (Burrus *et al.*, 2002).

Factors operating across HGT mechanisms

Restriction-modification systems

DNA restriction will constitute one of the first barriers to the survival of exogenous DNA within the cell. Restriction–modification (R–M) systems are ubiquitous in eubacteria and archaea. Most R–M systems comprise DNA methyltransferase and restriction endonuclease activities. The methyltransferase enables recognition of 'self' DNA by methylation of specific nucleotides within particular DNA sequences, whereas the endonuclease enzymatically cleaves foreign unmodified DNA. There are thousands of different R–M systems, which exhibit extensive diversity in their DNA sequence recognition specificities (Bayliss *et al.*, 2006). In the common type II R–M systems, specificity is conferred by an autonomous target recognition domain (TRD), which is present in both the methyltransferase and the endonuclease, enabling independent binding of each enzyme to the recognition site (Bujnicki, 2001). Type I systems contain a single specificity subunit that has two DNA-binding domains and is present in both enzymes. The gene encoding this subunit exhibits high levels of divergence within individual species and can undergo recombinatorial reassortment of the two DNA-binding domains resulting in enzymes with altered recognition sites (Dybvig *et al.*, 1998; Murray, 2000). Type III systems are less well characterized, but phylogenetic analyses indicate that regions likely to be involved in sequence specificity are under positive selection for variability and undergo intergeneric HGT (Bayliss *et al.*, 2006).

An efficient R–M system can drastically reduce the frequency of interspecies and even intraspecies HGT (Tock and Dryden, 2005; Hoskisson and Smith, 2007). However, several HGT mechanisms transfer DNA in a single-stranded form and ssDNA is thought to be rather refractory to the action of R–M systems (Thomas and Nielsen, 2005). In the case of phage or plasmid DNA that enters in a single-stranded form by transduction or conjugation, DNA becomes susceptible to restriction after synthesis of the second strand. Transformation is therefore the mechanism that is the least susceptible to restriction. For instance, *Streptococcus pneumoniae* contains R–M systems that restrict transduction among strains by factors of 10^{-5}–10^{-6}, whereas they only restrict plasmid transformation by half and they do not affect at all transformation with chromosomal DNA (Lacks and Springhorn, 1984). These different susceptibilities can be attributed to the fact that phage DNA is introduced into the cell in duplex form whereas transforming DNA enters as a single strand. In the case of transformed plasmids, complementing strand fragments from separately entering molecules form partially double-stranded intermediates that are then susceptible to cleavage by restriction enzymes (Saunders and Guild, 1981). Nevertheless, R–M systems in other organisms have been shown to affect transfer by transformation. Several restriction enzymes are known to mediate cleavage of ssDNA (Nishigaki *et al.*, 1985), and restriction of ssDNA has been shown to be a barrier to natural transformation in *Pseudomonas stutzeri* (Berndt *et al.*, 2003). Moreover, dsDNA has been detected after transformation of pathogenic *Neisseria* species (Jyssum *et al.*, 1971; Biswas and Sparling, 1981) and it has been proposed that this

genus may undergo transformation both by ssDNA, which could be used directly by RecA to mediate homologous recombination, and by dsDNA, which would require the DNA helicase activity of the RecBCD enzyme and would be susceptible to restriction (Taylor and Smith, 1980; Mehr and Seifert, 1998). Restriction is known to affect the population structure of *N. meningitidis*, which clusters into clades that are associated with different R–M systems, and transformation has been implicated in the generation of this structure because the density of DNA uptake sequences in different regions of the genome correlates strongly with the recombination rate (Budroni *et al.*, 2011). The capacity of restriction to limit intra- and interspecific transformation has also been demonstrated in *Staphylococcus aureus*, which contains several types of R–M systems that differ among isolates (Corvaglia *et al.*, 2010).

Interestingly, in spite of the protection that R–M can offer against infectious agents, several factors suggest that selective pressures to maintain these systems are sometimes alleviated. Many R–M systems display mechanisms that enable phase variation (PV), i.e. reversible, high frequency changes between on and off gene expression (Bayliss *et al.*, 2006) and R–M mutants that are hypersusceptible to the transfer of DNA from foreign sources are often found in nature (Sung and Lindsay, 2007; Corvaglia *et al.*, 2010). In strains of *Helicobacter pylori*, a type II R–M system is flanked by 80-bp direct repeats that facilitate its spontaneous deletion, but the system is easily reacquired by natural transformation (Aras *et al.*, 2001). In addition, many R–M systems are encoded in plasmids and can therefore also be easily lost and reacquired. These scenarios likely reflect the wide array of fitness effects potentially caused by foreign DNA from different sources or under different circumstances.

On the other hand, selective pressures clearly favour escape from R–M systems for MGEs. In plasmids and phage, mutation and selection frequently favour the loss of restriction sites or their disposition in arrangements that are less deleterious. For instance, the large genome of phage T7 is insensitive to the type III R–M system of *S. aureus*, because, even though it contains 36 recognition sites for this system's endonuclease, they are all present in the same orientation, whereas efficient restriction requires the head-to-head orientation of two consecutive sites for cleavage to occur (Corvaglia *et al.*, 2010). The selection pressure to loose recognition sites in MGEs likely results in a counteracting pressure for the evolution of new target recognition specificities in R–M systems, contributing to their large variability. Beyond recognition sequence loss and rearrangement, many phage and conjugative plasmids have specific mechanisms to evade the effects of host restriction systems, including the masking of restriction sites and the inactivation or modulation of R–M enzymes. For instance, in T-even phages DNA glucosylation is employed as a means of protection from most R–M systems, while T3 and T7 produce anti-restriction proteins that bind to type I restriction complexes and prevent them from binding to DNA (Murray, 2002). Interestingly, the structure of one of these proteins suggests that it neutralizes R–M complexes by mimicking the shape of the DNA substrate (Bandyopadhyay *et al.*, 1985; Atanasiu *et al.*, 2001). DNA mimicry has been shown to operate in other anti-restriction systems, such as the Ard (alleviation of restriction of DNA) proteins of conjugative plasmids (McMahon *et al.*, 2009). The proteins required for anti-restriction functions are often co-transferred with the MGE's DNA or produced early, so that they can anticipate the action of restriction systems. In the case of conjugative plasmids, this may entail transcription from special promoters recognized within secondary structures of the single DNA strand that enters the host cell (Bates and Sternberg, 1999), allowing for the accumulation of anti-restriction proteins before the transferred strand is converted into duplex DNA. Finally, conjugative plasmids

can also survive restriction by transiently saturating the system through the transfer of multiple plasmid copies or of extensive regions of chromosomal DNA (Matic *et al.*, 1996).

In addition to their protective role against infection, R–M systems may also have other relevant consequences for HGT, as restriction endonucleases generate recombinogenic DNA ends and could therefore potentiate the recombination of DNA fragments into the host chromosome (Matic *et al.*, 1996; Murray, 2002). Moreover, the fragmentation of incoming DNA may serve to separate potentially advantageous coding sequences from neighbouring deleterious ones (Milkman *et al.*, 1999).

CRISPR-based systems

A sophisticated strategy of defence against phages and plasmids has been recently uncovered in prokaryotes. This defence system features a unique type of repetitive DNA stretches, termed clustered regularly interspaced short palindromic repeats (CRISPRs). CRISPRs consist of identical repeats of DNA sequences separated by highly variable spacer sequences of phage or plasmid origin (Mojica *et al.*, 2005). Small RNAs produced from the spacer sequences guide CRISPR-associated (Cas) proteins to recognize and degrade, or otherwise silence, invading MGEs containing complementary sequences. In this way, CRISPR-Cas systems function as a sort of adaptive immunity that allows efficient recognition and neutralization of previously encountered foreign elements (Barrangou *et al.*, 2007; Marraffini and Sontheimer, 2008). There are multiple variations of CRISPR systems found in both eubacteria and archaea, mediated by largely distinct components and mechanisms (Terns and Terns, 2011). These systems are encoded by operons that have an extraordinarily diverse architecture and a high rate of evolution for both the *cas* genes and the unique spacer content. Classification systems are emerging to organize the different CRISPR-based systems of prokaryotes with basis on their sequence organization and evolution (Makarova *et al.*, 2011). In addition, the high variability of the CRISPR loci can be exploited for phylogenetic and evolutionary studies of closely related species and strains (Liu *et al.*, 2011) and for understanding the short-term dynamics of exposure to phages in bacterial populations and communities (Pride *et al.*, 2011). Moreover, given the importance of phage and plasmids in bacterial niche specialization, differences in CRISPR loci can be heavily involved in delineating intraspecific population structure and adaptive sublineage evolution (Fricke *et al.*, 2011).

Many questions still remain about the mechanisms of action of CRISPR systems and their regulation, but it is clear that these systems operate under tight regulatory control. In enteric bacteria, the expression of CRISPR arrays and associated *cas* operons is repressed by the histone-like nucleoid structuring protein (H-NS; Pul *et al.*, 2010), a well-known dual regulator of gene expression in Gram-negative bacteria. H-NS-mediated silencing can be counteracted by the transcription factor LeuO (Westra *et al.*, 2010). However, expression of *leuO* and *cas* genes is not induced by phage infection, suggesting that additional factors are involved in H-NS derepression. It is possible that incoming genetic elements compete with CRISPR/*cas* promoters for H-NS binding (Pul *et al.*, 2010; Westra *et al.*, 2010), given that H-NS has preference for binding AT-rich DNA and that horizontally transferred elements are normally enriched in ATs (Rocha and Danchin, 2002). Interestingly, H-NS generally binds and represses foreign DNA through a process known as xenogeneic silencing (Navarre *et al.*, 2006, 2007; Oshima *et al.*, 2006), and therefore can be thought of in itself as a self-protection mechanism, whose titration by large numbers of foreign AT-rich molecules

within the cell would represent an alert signal that would recruit the CRISPR systems of defence (Mojica and Diez-Villasenor, 2010).

Specific mechanisms through which phages may achieve resistance to CRISPR systems have not yet been described, but the high rates of sequence evolution by mutation or recombination undergone by many phages will facilitate their escape from memory-based immunity. Metagenomic analyses reveal that phage mosaicism in natural communities can be very high, involving mosaic fragment sizes smaller than the average CRISPR spacer, such that some phages in a given population may evade CRISPR blockage and continue to productively infect (Andersson and Banfield, 2008).

Recombination and DNA repair

Exogenous DNA that survives restriction and CRISPR systems may be integrated into the bacterial host chromosome, or another autonomous replicon, in order to persist in the recipient cell. By far, the most efficient mechanism of integration is homologous recombination (HR), but this mechanism is constrained by DNA sequence divergence. It has been demonstrated in various taxonomic groups that the efficiency of HR decreases with sequence divergence between host and incoming DNA (Shen and Huang, 1986; Majewski *et al.*, 2000; Meier and Wackernagel, 2005). Divergence affects the activity of recombination enzymes, particularly at the initial stage of the strand exchange process, which requires a minimum length of homology or minimum efficient processing segment (MEPS), below which recombination becomes inefficient (Shen and Huang, 1986). Divergence reduces recombination by reducing the number of MEPSs available for initiation (Shen and Huang, 1989). If initiated, strand exchange could proceed in spite of large numbers of nucleotide differences and even large insertions (Bianchi and Radding, 1983), but the methyl-directed mismatch repair system (MMRS) would detect the base-pair mismatches and unpaired bases in the resulting heteroduplex molecule and abort the recombination process (Shen and Huang, 1986; Worth *et al.*, 1994). Recombination between diverged sequences increases significantly in strains that are deficient in MMRS. For example, recombination in MMRS-deficient genetic backgrounds increases up to 10^3-fold between *Salmonella enterica* and *E. coli* (Rayssiguier *et al.*, 1989; Matic *et al.*, 1995) and 10^2- to 10^3-fold between serovars of *S. enterica* whose genomes differ only by 1–2% at the DNA sequence level (Zahrt *et al.*, 1994). Importantly, surveys of natural populations of *E. coli* have found that about 0.1–1% of cells are MMRS mutants and therefore have an enhanced chance of integrating DNA from different species into their genome by HR (Matic *et al.*, 1995). Another important factor affecting recombination is the SOS system, which actually has a facilitating effect that runs counter the inhibiting operation of the MMRS. The SOS system is a set of physiological responses that is induced by exposure of bacterial cells to a variety of conditions that damage DNA and/or interfere with its replication. The inducing signal is thought to be persistent ssDNA and, accordingly, the SOS is induced during conjugation, transformation, restriction of foreign or host DNA, transposition and whenever plasmid or phage DNA persists in single-stranded form. In fact, the SOS also appears to be induced under normal environmental conditions (Taddei *et al.*, 1995) and is required for efficient recombination during chromosomal conjugation and transformation because it stimulates overproduction of recombination enzymes. The degree of SOS induction is stronger in interspecific conjugation, probably because the difficulty in detecting MEPS to initiate recombination with divergent sequences results in longer persistence of ssDNA in the cell. Thus, the SOS system

acts as an inducible positive regulator of interspecies recombination, although it does not affect the activity of the MMRS, which remains a powerful inhibitor of recombination with divergent sequences even during strong SOS induction (Matic *et al.*, 1995). Consequently, the frequency of gene exchange by HR decreases with phylogenetic distance (Shen and Huang, 1986; Majewski *et al.*, 2000; Meier and Wackernagel, 2005).

Despite MMRS action, HR can participate in the chromosomal integration of foreign, non-homologous DNA. HR can proceed fairly efficiently *in vivo* between DNAs that differ by large insertions. If a segment of foreign DNA is embedded in sequences homologous to the recipient DNA, two HR events occurring in the flanks can effectively integrate it into the chromosome (Brigulla and Wackernagel, 2010). In natural transformation with homology-embedded foreign DNA, quantitative experiments revealed that homologous flanks of several hundred nucleotides suffice for integration and that longer flanks increase the integration frequencies by up to 100-fold (Simpson *et al.*, 2007). With the help of long homologous flanks, huge foreign DNA segments of up to 55 kb were integrated during transformation of *B. subtilis* (Itaya, 1999) and foreign DNA integrated by HR has been identified in the genome of *Mycobacterium* (Krzywinska *et al.*, 2004). The integration of foreign DNA can also proceed via homology-facilitated illegitimate recombination (IR), a combination of HR and IR. This synergy requires the presence of an anchor region, i.e. a single, short stretch of homology within the foreign DNA (Prud'homme *et al.*, 2002; de Vries and Wackernagel, 2002). The anchor region gets associated to the homologous recipient DNA by HR, and this step enhances the likelihood of IR in the adjacent foreign DNA. IR events at one or both flanks of the anchor can be stimulated, so that thousands of nucleotides can be integrated with anchor segments of just a few hundred bases. The efficiency of this mechanism varies greatly among species, but is always orders of magnitude below that of HR (Brigulla and Wackernagel, 2010). Mutants defective for recombination enzymes including RecBCD, SbcCD DNase, and RecJ DNase display up to 20-fold increased frequencies of homology-facilitated IR (Harms *et al.*, 2007; Harms and Wackernagel, 2008) and disruption of MMRS results in an increased net gain of foreign DNA and the appearance of hot spots for IR events (Meier and Wackernagel, 2005). Therefore, HR of homology-embedded foreign DNA and homology-facilitated IR mechanisms can promote the introgression into a species genome of sequences that have integrated into the chromosome of individual members of the species by any other means, including long genomic islands or prophages, and can be facilitated by genetic backgrounds deficient in MMRS and other enzymes involved in the process of recombination.

IR can also integrate foreign DNA into recipient chromosomes without help from homology regions or HR participation. A stretch of foreign DNA can be integrated by two IR events, although such integration is expected to be very rare considering that single IR events occur at frequencies of 10^{-6} to 10^{-10}. Nevertheless, experimental work has shown that such double IR events are detectable and the integration of 2 kb fragments of foreign DNA into the genome of *Acinetobacter* has been detected at frequencies of 10^{-13} (Hulter and Wackernagel, 2008). The generation of mosaics between host and phage DNA has also recently been shown to occur by IR events (Morris *et al.*, 2008).

In addition to HR and IR, other integration mechanisms can also operate for foreign DNA. More than a hundred different enzymes, nearly all encoded by MGEs, recombine DNA at short specific nucleotide sequences, without the long stretches of homology required for HR, by means of a process involving DNA cleavage and reunion without DNA

synthesis. Site-specific recombinases act as single enzymes or in complexes with other proteins and can catalyse the integration, excision, and inversion of DNA segments. They are responsible for the insertion of temperate bacteriophages and ICEs into new locations and for the continuous exchange of genes among these elements and bacterial chromosomes (Brigulla and Wackernagel, 2010).

Barriers and fitness costs for expressing foreign genes

Genes from different species have different GC contents and codon usage biases and several aspects of gene expression are highly tuned to the particular sequence characteristics of each genome. Such species-specific tuning can represent a substantial barrier to interspecific gene exchange by compromising foreign gene expression in different ways. One of these is represented by the histone-like nucleoid-structuring protein (H-NS), which is one of the major protein components of the nucleoid structure in bacteria and binds large regions of DNA, down-regulating gene expression, with a marked preference for AT-rich sequences. In general, horizontally transferred sequences tend to be AT-rich, and, therefore they are often recognized by H-NS and transcriptionally repressed, in a process termed xenogeneic silencing (Lucchini *et al.*, 2006; Navarre *et al.*, 2006, 2007). The expression of foreign genes can also be compromised because the host RNA-polymerase and/or transcription factors may not efficiently recognize the promoters and other regulatory sequences evolved in a different genomic background. At the translational level, expression can be affected by the codon biases of foreign genes, especially for those genes that were highly expressed in their genomes of origin. Such genes utilize the most species-biased sets of codons, because they are most strongly selected for translational efficiency and thus for using the codons that correspond to the most abundant tRNAs in the cell, which vary across species (Ikemura, 1985; Sharp and Li, 1986; Kanaya *et al.*, 1999; Rocha, 2004; Tuller, 2011).

Even when a foreign gene could be effectively expressed in a novel genome, the fitness cost of expressing it might result in selection against the recipient cell. If the gene product represents no selective benefit, cells that express it will undergo unnecessary expenditures in transcription and translation. Of course, foreign gene expression could also have negative effects on the functioning of the cell, especially if the expressed proteins affected the operation of basic cell processes. In particular, proteins that are highly interactive can interfere with resident protein complexes or protein interaction networks, due to the generation of stochiometric imbalances or to suboptimal activity in comparison with the native homologues. The fitness effects ensuing from the interspecific transfer of different classes of genes were further discussed in Chapter 1.

Interactions among MGEs

Presence of a given MGE in a host cell can affect in a positive or in a negative manner the likelihood that another element is acquired or maintained. A classical example of negative interaction is phage superinfection immunity, whereby gene expression of an incoming phage is impeded by repressors encoded by a similar prophage. Among conjugative plasmids, transfer into a recipient cell containing the same or a closely related plasmid is inhibited by a mechanism known as entry or surface exclusion. All investigated conjugative plasmids contain at least one entry exclusion gene (Garcillán-Barcia and de la Cruz, 2008). This exclusion system is believed to confer an evolutionary advantage to the plasmid

as it frees the plasmid from competition at segregation during cell division, and protects the plasmid-bearing cell from too many conjugation events (Thomas and Nielsen, 2005; Garcillán-Barcia and de la Cruz, 2008). Laboratory experiments suggest that surface exclusion systems can inhibit the entry of a similar plasmid by up to 500-fold, as in the case of the R388 plasmid discussed above (Garcillán-Barcia and de la Cruz, 2008; Perez-Mendoza and de la Cruz, 2009). Moreover, even if entry exclusion fails, the long-term coexistence of plasmids sharing replication and partitioning mechanisms is unsustainable, which serves as a basis for their classification in incompatibility (Inc) groups. Similarly, some ICEs, although not all, affect the likelihood of acquisition of closely related elements (Burrus *et al.*, 2002). Negative interactions can also occur among MGEs of very different types. For instance, the conjugative *Lactococcus* plasmid pNP40 protects cells from infection by phages via several mechanisms that include prevention of phage DNA entry into the cell as well as a R–M system (O'Driscoll *et al.*, 2006). In addition, host factors can modify the interaction between MGEs. For instance, the presence of incompatibility among similar plasmids can depend on the *E. coli* strain serving as host (Grant *et al.*, 1980).

On the other hand, the presence of different MGEs in a host cell can also enable or enhance an element's capacity to transfer, such as in the widespread phenomenon of mobilization of non-conjugative plasmids by conjugative elements. Another important synergy among MGEs derives from the fact that the ability to generate biofilms and other multicellular bacterial structures is often encoded in plasmids (Ghigo, 2001; Norman *et al.*, 2008). As biofilms are ideally suited to the exchange of genetic material of various origins, the acquisition of a biofilm-inducing plasmid in a bacterial community will affect the subsequent likelihood of transmission of other conjugative and mobilizable MGEs.

Coexistence of MGEs in the same cell can of course also result in the exchange of all sorts of genes among different elements, thus altering the potential availability of shuffled genes to new recipient hosts. In particular, novel properties affecting host range can appear by recombination among different types of MGEs. For instance, conjugative plasmids have been shown to appear by co-integration of ICEs and non-conjugative plasmids followed by deletions and other genomic rearrangements (Gasson *et al.*, 1992; Salyers *et al.*, 1995a,b). Similarly, ICEs can be turned into plasmids capable of sustaining their own replication by co-integration of plasmid-derived replication origins. For instance, *L. lactis* can harbour an ICE known as *sex factor*, whose transfer is generally limited to intraspecific strains. However, this ICE can be transmitted to a different Firmicutes genus if a plasmidic origin co-integrates in the circular intermediate that forms after excision from the host chromosome; the sex factor contains a group II intron which may in this way transfer between different genera (Belhocine *et al.*, 2007).

The shuffling of genes among MGEs is facilitated by several factors. First, most elements are organized in a highly modular fashion, as already mentioned above for plasmids and ICEs (Burrus *et al.*, 2002; Weaver *et al.*, 2009). Modularity and recombination also play a major role in phage evolution (Lucchini *et al.*, 1999) and recombination across MGEs of different types, via the exchange of modules or the co-integration of an element within another, is a common phenomenon. Plasmids, for instance, usually carry insertions of one or more transposons or other MGEs and the incorporation and expelling of such elements occur more frequently than do changes in the core plasmid backbone (Norberg *et al.*, 2011). For their part, ICEs and related mobilizable elements comprise functional modules that can be derived from phage, plasmids or transposons and it has been proposed that the combination

of these modules generates, not discrete classes, but a continuum of mosaic MGEs (Osborn and Böltner, 2002). Further, MGEs can contain specialized systems for gene integration and expression, known as integrons. Integrons are genetic elements able to acquire and rearrange open reading frames (ORFs) embedded in gene cassette units and convert them to functional genes by ensuring their correct expression. They contain gene cassettes of heterogeneous origins probably collected successively in different genomics backgrounds. Integrons are rendered mobile by virtue of their association with transposons, often themselves carried on conjugative plasmids (Cambray *et al.*, 2010). In fact, transposon-associated integrons are one of the major conveyors of antibiotic multiresistance across Gram-negative and, to a lesser extent, Gram-positive bacteria (Partridge *et al.*, 2009). Non-mobile integrons are also present in the chromosomes of hundreds of bacterial species across diverse phyla and it is now thought, based on phylogenetic analysis, that the chromosomal integrons are the source of the integrons found in association with MGEs (Mazel *et al.*, 1998; Cambray *et al.*, 2010).

Overall relationship between phylogenetic distance and HGT

Although most gene transfers take place between closely related organisms (Lawrence and Hendrickson, 2003) with compatible gene contents, many studies have revealed that HGT events can and do occur between evolutionarily distant taxa. In a survey of curated protein domains in the Pfam database ($n = 7677$ families), Choi *et al.* (2007) found that horizontal gene transfer decreased the higher the taxonomic range of the species considered regardless of the biological domain (Bacteria, Archaea or Eukarya). The extent of HGT varied from 1.1% (74 families) at the second, to 5.3% (365 families) at the third and 9.7% (667 families) at the fourth taxonomic ranges. In light of the wide breadth of taxonomic ranges actually covered in these three levels of the Pfam database (for instance, the third level comprised from phylum to family), it is not possible to derive estimates of HGT at recognizable taxonomic categories but, nevertheless, these authors concluded that HGT will have little impact on the construction of organism phylogenies, especially when phylogenomic or methods based on whole genome sequences are used.

Coscollá *et al.* (2011) studied what fraction of the genome of a single species results from strictly vertical inheritance and which derives from HGT events. The phylogenomic analysis of 1700 genes in four genome sequences of *Legionella pneumophila* revealed that as much as 42% of these genes were derived from non-γ-Proteobacteria, the class where *L. pneumophila* belongs. This large proportion of horizontally transferred genes is most likely facilitated by the intracellular, although transiently, lifestyle of *Legionella* in the cytoplasm of amoebas, which provide one of the environments most facilitating of lateral transfers (see above). In this same study, the authors analysed the relationship between number of HGT events and phylogenetic distance. Despite environmental facilitation, a clear negative correlation between these two variables was observed, thus indicating that horizontal transfers not only depend on opportunity but also on other limits some of which are clearly associated to evolutionary distance.

An interesting relationship between HGT from distant taxa, genome size, functional class and ecology has been suggested by Cordero *et al.* (2009). They analysed 333 complete bacterial genomes and determined the distribution of protein families in close and distant HGTs along with other relevant features. They found that large genomes tended to have a

larger contribution of evolutionarily distant genes, which were enriched in two functional categories, transcription regulation and defence mechanisms. The former category includes one-component systems, which incorporate DNA binding and substrate recognition domains, thus linking environmental stimuli and regulatory responses in a single protein. The most relevant component of HGT in the defence mechanisms corresponds to transporters involved in extrusion of toxic compounds and to R–M systems. In order to explain these patterns, Cordero *et al.* (2009) put forward a hypothesis in which complex environments would be selective for larger gene repertoires, in order to cope efficiently with the multiplicity of interactions in species-rich communities. The increase in gene repertoire seems to be mainly due to lateral transfer (Kuo and Ochman, 2009). The presence in these environments of phylogenetically diverse species along with the need to increase the gene repertoires of the genomes involved therein would lead to a circular process: more complex interactions in species-rich communities would increase the demand for new genes, which might be covered by incorporating transfers from distant species, resulting in more complex species and even more complex environments.

Conclusions

The flow of genes across taxa through HGT is enabled by the existence of complex networks of loopholes across transfer barriers, often provided by a small fraction of the individuals in an otherwise inaccessible population. HGT networks are fluid and highly evolvable, as is evidenced by the large intraspecific variability often observed in terms of the capacity of strains to donate or receive DNA by means of different mechanisms, and in the establishment of coevolutionary arms races among, particularly, bacteria and phage. One of the most important properties of HGT networks is their gene specificity, i.e. the fact that the genes of a potential donor genome will have an unequal likelihood of passing through or being incorporated in a given recipient. This gene specificity should have consequences for prokaryotic evolution at several different levels. From a gene-centric view, the long-term selective pressures that will operate on a gene will depend on the number and type of genomic backgrounds in which it might find itself and on the frequency of exchanges among them. At the other extreme, the existence of gene-specific transfer networks will create groups of organisms that will be partially related through gene sharing, but the relatedness among individuals will differ among genetic loci. Beyond the impact that they may have on prokaryotic phylogeny and species concepts, the specific ways in which HGT networks will affect genic, organismal and phyletic evolution need to be quantitatively explored, as they could result in unforeseen evolutionary dynamics in the prokaryotic world.

Acknowledgement

We would like to thank Yvonne Vallès for her help with the references cited in this chapter.

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