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MECHANISMS OF, AND BARRIERS TO, HORIZONTAL GENE TRANSFER BETWEEN BACTERIA

Christopher M. Thomas and Kaare M. Nielsen‡*

Abstract | Bacteria evolve rapidly not only by mutation and rapid multiplication, but also by transfer of DNA, which can result in strains with beneficial mutations from more than one parent. Transformation involves the release of naked DNA followed by uptake and recombination. Homologous recombination and DNA-repair processes normally limit this to DNA from similar bacteria. However, if a gene moves onto a broad-host-range plasmid it might be able to spread without the need for recombination. There are barriers to both these processes but they reduce, rather than prevent, gene acquisition.

The first evidence that horizontal gene transfer (HGT) could occur was the recognition that virulence determinants could be transferred between pneumococci in infected mice, a phenomenon that was later shown to be mediated by the uptake of the genetic material DNA in a process called transformation[1]. The subsequent identification of gene transfer mediated by both plasmids and viruses and the recognition of transposable elements provided the stepping stones to our current picture of gene flux and the importance of mobile genetic elements[2]. Compositional analysis has revealed that considerable proportions of most bacterial genomes consist of horizontally acquired genes[3].

Many horizontally acquired genes are likely to cause deleterious effects in the chromosome of the bacterial recipient. Therefore, these bacteria will be lost from the population over time in the same way that deleterious mutations are lost. Some horizontal acquisitions might be effectively neutral, and their survival will depend on chance events. However, horizontally acquired chromosomal DNA that confers a selective advantage to the host, or mobile genetic elements that encode their own transfer and maintenance functions, have the potential to spread rapidly within a bacterial population. Where HGTs succeed between distantly related organisms, the genes most likely to be involved tend to belong to the simplest sets of functional networks[4]. Therefore, informational genes of the central cellular machinery such as DNA replication, transcription or translation tend not to spread rapidly, even if they confer antibiotic resistance, compared for example to single-function-resistance determinants such as β-lactamases or aminoglycoside-modifying enzymes. However, the nature of the transfer mechanism can also determine the organisms and genes that are most often involved. The purpose of this review is to describe some of the mechanisms that lead to horizontal gene acquisitions with a view to understanding what limitations there are to these processes. The journey therefore starts with DNA in a potential donor and considers the various steps (as outlined in FIG. 1) until the DNA becomes a functional part of a recipient genome. We focus on natural transformation and conjugation and consider single-cell events only. The subsequent population-dynamic aspects of HGT events are dealt with elsewhere (see the article by J.P. Gogarten and J.P. Townsend in this issue)[5].

Natural transformation

Natural transformation — the stable uptake, integration and functional expression of extracellular DNA that can occur under natural bacterial growth conditions — is the only mechanism that can potentially explain how bacteria acquire DNA from foreign species beyond the host range of mobile genetic elements or bacteriophages.

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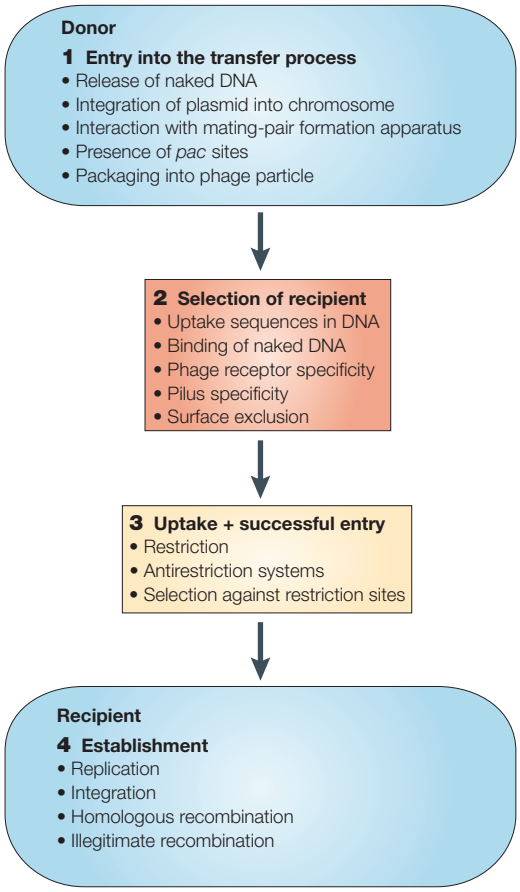

Figure 1 | **The process of horizontal gene transfer.** A schematic outlining the stages through which DNA must go on its journey from donor to recipient bacteria. The process begins with DNA in a potential donor cell becoming available and ends when this DNA becomes a functional part of a recipient cell's genome.

For natural transformation to occur, bacterial cells must first develop a regulated physiological state of COMPETENCE, which has been found to involve approximately 20 to 50 proteins. With the exception of *Neisseria gonorrhoeae*, most naturally transformable bacteria develop time-limited competence in response to specific environmental conditions such as altered growth conditions, nutrient access, cell density (by quorum sensing) or starvation. The proportion of bacteria that develop competence in a bacterial population might range from near zero to almost 100%. As the growth environments and factors that regulate competence development vary between bacterial species and strains[6], there is no universal approach to determine if a given bacterial isolate can develop competence as a part of its life cycle. To the extent investigated, the proportion of bacteria found to be naturally transformable is approximately 1% of the validly described bacterial species[7]. The ability to take up naked DNA by natural transformation has been detected in archaea and divergent subdivisions (phyla) of bacteria, including representatives of the Gram-positive bacteria, cyanobacteria, *Thermus* spp., *Deinococcus* spp., green sulphur bacteria and many other Gram-negative bacteria[8,9]. Many human pathogenic bacteria, including representatives of the genera *Campylobacter*, *Haemophilus*, *Helicobacter*, *Neisseria*, *Pseudomonas*, *Staphylococcus* and *Streptococcus*, are naturally transformable[9]. The conserved ability to acquire DNA molecules by natural transformation among a broad range of bacteria indicates that the genetic trait is functionally important in the environment, enabling access to DNA as a source of nutrients or genetic information. Prerequisites for natural transformation include the release and persistence of extracellular DNA, the presence of competent bacterial cells and the ability of translocated chromosomal DNA to be stabilized by integration into the bacterial genome or the ability of translocated plasmid DNA to integrate or recircularize into self-replicating plasmids (FIG. 2).

Release of extracellular DNA in the environment. Natural transformation relies on bacterial exposure to extracellular DNA molecules in the environment. DNA continually enters the environment upon release from decomposing cells, disrupted cells or viral particles, or through excretion from living cells. The release of intact DNA from decomposing cells depends on the activity and location of nucleases and reactive chemicals. Active excretion of DNA has been reported for many genera of bacteria, including *Acinetobacter*, *Alcaligenes*, *Azotobacter*, *Bacillus*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Streptococcus*[8–10]. For instance, extracellular DNA has been found at concentrations of up to 1–3 μg per ml in liquid cultures of an *Acinetobacter* sp. and *Bacillus subtilis*[11] and up to 780 μg per ml in cultures of the environmental isolate *Pseudomonas aeruginosa* KYU-1 (REF. 12). Recently, extracellular DNA has been identified as an important component in biofilm formation[13]. Nevertheless, the extent of, and role of, active release of DNA by bacteria in natural, nutrient-limited habitats remains to be fully understood.

Passive release of DNA from dead bacteria occurs after self-induced lysis, a process that results in broken cell walls and membranes and the subsequent exposure to, and release of, cytoplasmic contents, including DNA, in the environment[14]. Pathogenic microorganisms can also undergo lysis caused either by the host immune system or the antibiotic treatment of infections. From studies of ^{14}C-labelled *Escherichia coli*, it has been estimated that between 95% and 100% of the bacterial DNA is released after contact with the immune system[15]. Most of this DNA is probably degraded by DNases present in human serum and plasma. In one study, the mean DNase activity of 50 patients destroyed 90% of the added DNA of *Haemophilus influenzae* within a few minutes[16]. A different study, however, reported longer persistence times for both chromosomal and plasmid DNA in serum[17] — large plasmids and chromosomal DNA were substantially degraded after a 4-hour exposure of a serum-sensitive *E. coli* strain, but smaller plasmids (pBR322 and

COMPETENCE
The ability of bacteria to take up extracellular DNA.

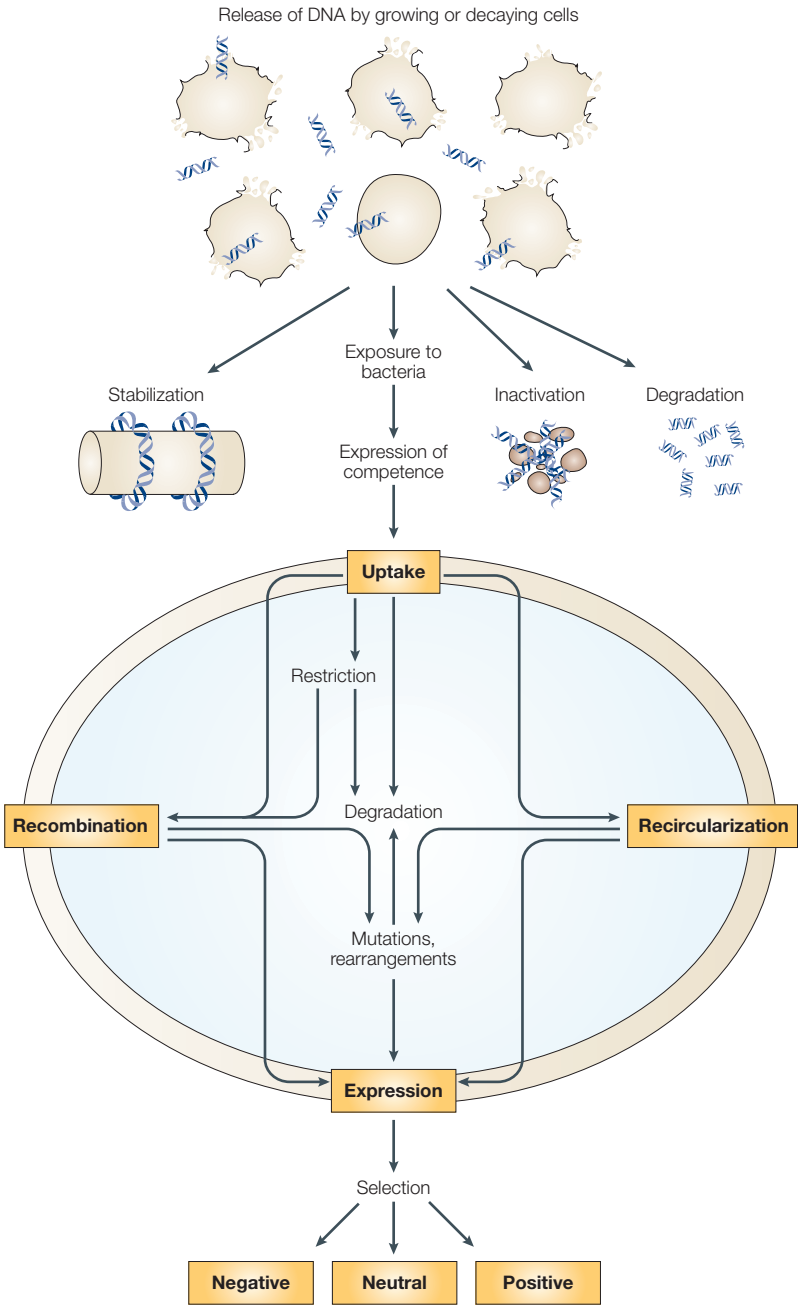

Figure 2 | **The natural transformation of recipient bacteria and selection of transformants.** The steps involved in this process include the release of extracellular DNA into the environment and the uptake of DNA into the cytoplasm of the recipient bacterial cell that has developed a regulated physiological state of competence. Following uptake, for the transferred DNA to persist it must integrate into the bacterial genome through homologous recombination or by sequence-independent, illegitimate recombination. Plasmids that succeed in reconstituting a replication-proficient form do not need to integrate into the host genome. Modified with permission from REF. 159 © (1998) Blackwell Publishing, and REF. 160 © (1998) Elsevier.

RSF1030) remained largely intact. These studies show that bacterial DNA can persist in blood long enough for natural transformation of infectious bacteria to occur. In addition to DNA released by pathogenic and commensal bacteria, DNA present in the intestinal tract can temporarily enter the bloodstream and reach various organs in mammals[18].

TRANSFORMATION FREQUENCY
The number of bacteria carrying the horizontally acquired DNA divided by the total number of bacteria exposed, per given time unit.

Stability of extracellular DNA in the environment. The persistence of extracellular DNA will determine the bacterial exposure time, and therefore the natural TRANSFORMATION FREQUENCY. The degradation kinetics of extracellular DNA vary considerably depending on environmental conditions[19–23]. Detection of ancient DNA in conserved specimens that are estimated to be several million years old illustrates the importance of environmental conditions for DNA preservation and degradation to occur[24,25]. In soil and sediments, approximately 1 µg extracellular DNA can be recovered per g material[26], and in fresh and marine water approximately 0.03 to 88 µg dissolved DNA can be found per litre[27,28]. Investigations of DNA stability in plant material and plant-based feed have recovered minor fragments of DNA from the mammalian intestinal system[18,29,30], faeces[18,29,30] and ensilaged plants[30,31] for up to several weeks, and from residues of field-grown plants for up to several years after cultivation[21,32–34]. Experimental investigations adding pure nucleic acids into soil[35,36], water[37,38], human saliva[39], silage[40] or various types of food and feed[41,42] have shown that the DNA is not immediately degraded and will persist for hours to days, as measured in natural transformation assays.

Uptake of DNA into the bacterial cytoplasm. Upon exposure to competent bacteria, the extracellular DNA binds non-covalently to sites present on the cell surface. The number of binding sites has been estimated for only a few bacterial species and ranges from 30 to 80 in *Streptococcus pneumoniae* and *Acinetobacter baylyi*[43,44]. The subsequent DNA-translocation process varies among bacteria and remains to be fully characterized[45,46]. For an excellent recent review of DNA uptake during natural transformation, we refer to Chen and Dubnau[47]. Double-stranded DNA is converted to single-stranded DNA during translocation across the inner membrane[47]. Some competent bacterial species, for example, *N. gonorrhoeae* and *H. influenzae*, are selective in the DNA they translocate across the membrane, whereas most other species take up DNA independently of its sequence[9]. Sequence-specific uptake is based on short (~9–11 bp in length), interspersed (~4–5 kb apart) nucleotide motifs in the bacterial genomes. The uptake of plasmids and transfer of chromosomal DNA between distantly related genera might therefore be highly restricted in these particular species, although it remains to be determined to what extent sequence-determined surface discrimination is absolute and at what level leakage occurs[9]. Most competent bacteria with a sequence-independent DNA-uptake mechanism can translocate both plasmids and chromosomal DNA across their membrane(s). However, owing to the enzymatic DNA cleavage and conversion to single-strand form that occur during the translocation process in natural transformation, the uptake of plasmids requires complex steps to reassemble a circular duplex molecule in the recipient cytoplasm. This normally requires either the presence of the plasmid in the recipient already, for the plasmid DNA to be multimeric or for

it to enter in multiple copies. Therefore, natural transformation with plasmids is usually less efficient than with chromosomal DNA fragments[48]. The uptake of DNA by competent bacteria occurs rapidly *in vitro*, at about 100 bp per second in *S. pneumoniae*[49] and 60 bp per second in *A. baylyi*[43]. Internalized DNA will normally persist only transiently in the bacterial cytoplasm owing to the inability of DNA fragments to replicate during bacterial cell division. If the DNA is reassembled as double-stranded DNA in the cytoplasm, restriction enzymes might cause rapid fragmentation. However, because natural transformation usually involves single-stranded DNA and recombination, restriction is not considered an important barrier for it to occur[50].

Recombination with the host genome. With the exception of plasmids that succeed in reconstituting a replication-proficient form, the horizontally transferred DNA transiently present in the bacterial cytoplasm must integrate into the bacterial genome to persist for many generations. For HOMOLOGOUS RECOMBINATION, depending on the system, the incoming DNA must contain regions between 25 to 200 bp in length of high similarity to the recipient genome. These regions will initiate DNA pairing and strand exchange. Under optimal *in vitro* conditions, it has been estimated that 0.1% of internalized DNA fragments are successfully recombined in *A. baylyi*, whereas up to 25–50% of the internalized DNA fragments are recombined in *B. subtilis* and *S. pneumoniae*[43]. DNA-sequence analysis indicates that the recombination rate in chromosomal housekeeping genes is of the order of mutation frequencies in bacteria within natural populations of *E. coli*, *Neisseria* spp. and *Streptococcus* spp.[51,52] Probabilistic calculations indicate that the presence of short regions of high DNA similarity will be limited to DNA fragments of less than 25 to 30% divergence[53]. Many studies confirm that recombination occurs between chromosomal DNA that is less than 25% divergent[54–58]. In *B. subtilis*, *E. coli* and *S. pneumoniae*, a log-linear decrease in recombination frequencies with increasing sequence divergence has been established[55,57,58].

As sequence divergence increases between the participating DNA molecules, constraints to protein functionality probably lower the number of allelic replacements that will be neutral or positively selected within a bacterial population[53]. Whereas fragments of DNA of a size that could encode entire protein-coding regions are recombined by homologous recombination at frequencies that have been estimated to be proportional to sequence divergence, there are few estimates for the frequency at which short linear DNA fragments of a few bp to less than 200 bp recombine with bacterial chromosomes[59]. Recombination events resulting in nucleotide changes of only a few bp can be difficult to distinguish from genetic changes arising from sequential mutations, and the mechanism responsible, HGT, is therefore easily overlooked unless larger DNA-sequence datasets are available for comparative analysis[52,60]. The need for sequence similarity for integration might be alleviated by sequence-independent, illegitimate recombination events[61]. Several genes have been identified as controlling illegitimate recombination through double-stranded breaks and end-joining in *E. coli*[60]. However, DNA acquisition by such processes apparently applies more to integration of circular DNA than linear fragments. Most laboratory studies suggest that the chromosomal uptake of unrelated DNA fragments in wild-type bacteria through illegitimate-recombination events are low-frequency events[56,62]. This is also expected to limit the bacterial loss of fitness that is unavoidable if recombinations with foreign chromosomal DNA were frequent[63].

Acquisition of DNA through additive integration. In addition to the recombination processes that lead to simple DNA replacements that preserve the size and functionality of the recombined area, natural transformation can also lead to illegitimate recombination events (see above) or homology-based recombination events (see below) that lead to the acquisition of additional DNA material from the donor DNA molecules.

Recombination that occurs between two circular molecules or between a circular molecule and the bacterial chromosome leads to additive integration[64]. This process is based on single crossovers at short regions with high DNA-sequence similarity and leads to the fusion of plasmids or other circularized DNA molecules, or the integration of circular DNA molecules into the bacterial chromosome. Studies in *E. coli* show that homology-based recombination occurs in plasmids when homologous sequences of 25 bp and higher are present. Increasing the length of DNA homology increases recombination rates by several orders of magnitude, the recombination rates reaching a plateau for homologous regions longer than 200 bp[65]. Owing to the short stretches of DNA-sequence similarity that are required to initiate the events, circular DNA molecules have the potential to be some of the most promiscuous recombinogenic states of all DNA[66], although this is not normally the state of DNA entering cells through natural transformation systems.

Recombination that occurs between linear DNA and chromosomal DNA molecules can also lead to the additive integration of DNA[67]. In this process, once recombination has been initiated in a single region of high DNA similarity, the strand exchange extends into regions of little or no nucleotide-sequence similarity, resulting in substitution of DNA sequences or often the integration of additional DNA sequences. Recombination processes that extend into areas with little similarity have been named homology-facilitated illegitimate recombination (HFIR)[68,69]. For instance, in *A. baylyi* and *S. pneumoniae*, single regions of high nucleotide-sequence similarity (~200 bp in length) have been shown to initiate recombination events that lead to the additive integration of >1000-bp-long heterologous DNA fragments into the bacterium's

HOMOLOGOUS RECOMBINATION
Recombination that depends on extensive segments of high sequence similarity between two DNA molecules.

genome[70,71]. Some of the host DNA might be deleted in the integration process. HFIR events, based on initial recombination in a single region, occur at frequencies several orders of magnitude below those observed for homologous DNA.

Natural transformation of bacteria resulting in the acquisition of chromosomal DNA fragments of several kb can, however, occur at high frequencies (up to 1% of transformants) in a bacterial population when two flanking regions with high sequence similarity are present[36]. The mechanism of additive integration of DNA, based on two flanking regions of high DNA similarity to initiate the recombination process, has been the basis for the many recent studies that have reported that bacteria can take up fragments of transgenes from plants *in vitro*[33,70], in soil[72] or within infected tobacco plants[73]. In these studies, the recipient bacteria harboured DNA with sequence identity to parts of the specific marker genes monitored for HGT. These latter studies show that there are no specific barriers that prevent competent bacteria from using genetic information present in released DNA from highly unrelated organisms if stabilization and expression is enabled. From these observations, and the studies presented in the previous paragraphs on the processes leading to DNA release and stability, it can be inferred that bacterial genomes are transiently exposed to DNA of other cells at appreciable frequencies in nature.

Naturally occurring bacterial strains with altered recombination potential. The dependence on DNA-sequence similarity for recombination to occur between species is relaxed in some bacterial mutator strains[74]. Mutator phenotypes can arise transiently in response to altered growth conditions[75], or more stably through mutations in genes that belong to the DNA-repair and maintenance machinery[54,74,76]. Heritable mutator strains have been found in natural populations of *E. coli*, *Salmonella* spp., *P. aeruginosa* and *Neisseria meningitidis* at proportions between 1% to 20% (REFS 76,77) and in experimentally evolving populations of bacteria. Some mutator strains recombine at elevated frequencies with divergent DNA, particularly those with mutations in the methyl-directed DNA-repair genes (for example, the *mutS* gene in *E. coli*)[55,57,58,68,78], and it has been proposed that such phenotypes have access to a different gene pool than non-mutator strains[53]. The environmental distribution, dynamics and contribution of mutator phenotypes to bacterial adaptation are currently being addressed.

Conjugative transfer

Whereas plasmids might be taken up by natural transformation, a process more specifically linked to plasmid acquisition is conjugative transfer. Conjugative transfer is mediated by cell-to-cell junctions and a pore through which DNA can pass, although the nature of these structures remains elusive (FIG. 3). Conjugative transfer systems are frequently associated with plasmids, probably because, to evolve, they would normally need the genetic element of which they are a part to be

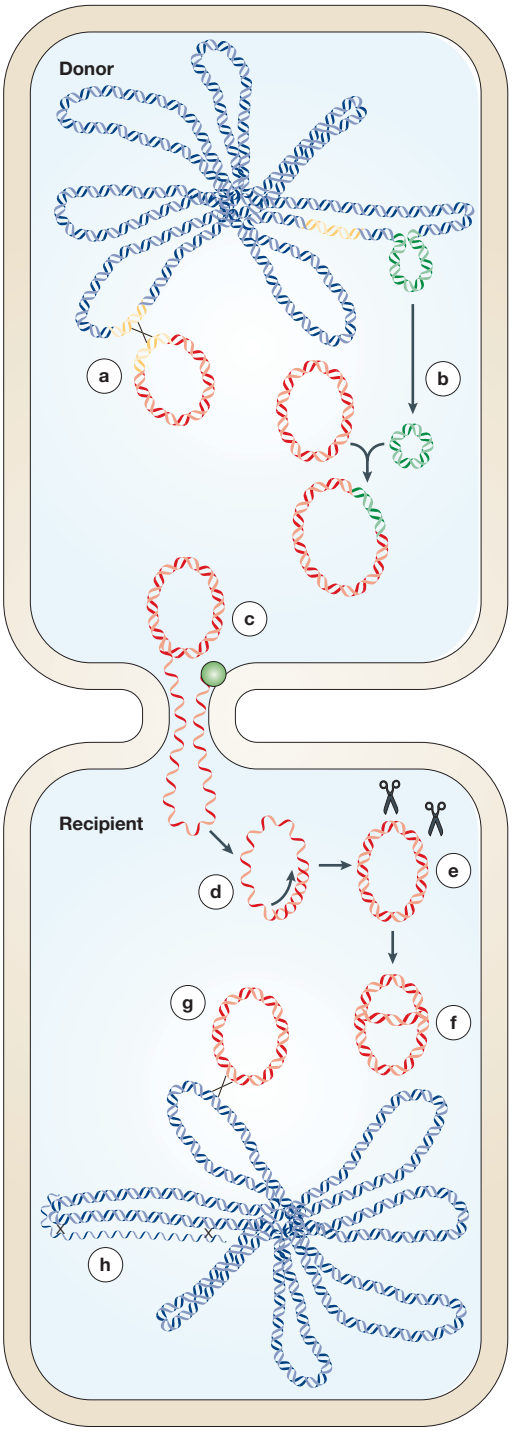

Figure 3 | **Overview of plasmids and conjugative transfer in the horizontal spread of genes.** In the donor, the events depicted are: **a**, integration of the plasmid into the chromosome by recombination between insertion sequence elements; **b**, movement of a transposable element through a circular intermediate from the chromosome to the plasmids; **c**, initiation of rolling-circle replication at the mating-pair apparatus. In the recipient cell, the events depicted are: **d**, recircularization; **e**, attack by restriction endonucleases (scissors); **f**, replication; **g**, integration into the chromosome by an illegitimate Campbell recombination; **h**, recombination between transferred chromosomal DNA and the resident chromosome.

Box 1 | **Transposable elements and the transfer of DNA**

The simplest transposable elements are insertion sequence (IS) elements, which can be as short as 600–700 bp, simply encoding a transposase. The presence of several closely related IS elements in a genome of a bacterium allows homologous recombination between unrelated elements, so long as each of the elements carries a copy of the same IS element. This has long been known as the mechanism whereby the *Escherichia coli* sex factor F integrates into the chromosome by recombination between copies of IS*2* or IS*3*, so that the junction between F and the chromosome is defined by the presence of the IS element. Subsequent recombination between other copies of that element can then result in what are called F' elements, which now contain not only all or most of the plasmid, but also a section of the chromosome.

completely transferred to the recipient before the genes for the conjugative system can be reconstituted in an active form. Therefore, the transfer systems that are most widespread are those on relatively small genetic elements — plasmids — that can be transferred quickly. By contrast, a whole chromosome can take more than 1 hour to be transferred, and this process will rarely go to completion because the interbacterial junctions will break down. Those conjugative systems not associated with plasmids tend to be part of elements that excise from chromosomes or other parental elements prior to transfer and reintegrate after transfer by mechanisms that do not rely on homologous recombination[79]. This section will focus primarily on plasmids because, at its simplest, a plasmid is an autonomously replicating genetic element that, if it enters as a double-stranded element, can remove the need for a foreign gene to integrate into the recipient chromosome to become established. However, the existence of conjugative apparatuses can result in transfer of not only whole plasmids, but also extensive sections of any other genetic element, including chromosomes, that have become physically part of the genetic unit that contains the transfer origin that initiates the movement of DNA between paired bacteria.

Interaction of conjugative elements with the other genetic elements in the cell. Conjugative elements can facilitate the transfer of additional DNA, either by picking up genes which they then carry to recipients or by integrating into other elements, allowing them to benefit from the self-transmissibility of the element. Transposable elements are the best-known facilitators of such interactions (BOX 1). That this is a general principle resulting in constant flux within the genome has been shown in *Rhizobium* species by PCR with primers designed to survey products of recombination mediated by INSERTION SEQUENCE (IS) elements and other repeated sequences[80]. With the knowledge of a genome sequence, primers can be designed for the unique sequences that flank known IS elements. Recombination events will bring different flanking regions together, and these can be detected by PCR. The results show that, in a large bacterial population, many of the possible products of recombination between repeated elements exist at any one time, including those with plasmids integrated into the chromosome[81]. The reason why, in practice, genomes seem to be reasonably stable might be the need for a considerable growth advantage for a variant genome structure to sweep through a population. It has not yet been established whether there are important growth-rate differences between the different genomic permutations. In the absence of such differences, the long timescales involved would make experimental investigation of population changes unfeasible.

Not all self-transmissible plasmids carry transposable elements that are already well distributed throughout bacterial genomes. The broad-host-range IncP-1 plasmid R68 carries IS*21*, which occurs in less than one-fifth of bacterial divisions[82]. R68 was found to be a poor mobilizer of chromosomal DNA, but activation of IS*21* to become more transpositionally active resulted in a plasmid that promotes movement of chromosomal genes at higher frequency[83]. This activation occurred through a direct duplication of IS*21*, creating a promoter across the junction between the end of one copy of IS*21* and the beginning of the next. This promoter increases the transcription of the transposition genes *istA* and *istB*, so that the transposition frequency rises[84]. Recent work has shown that many IS elements might normally transpose in two steps[85]. First, a TRANSPOSASE catalyses the generation of a free copy of the element as a circle at low frequency[86]. The ends of the autonomous element are therefore joined to create a stronger *tnpA* promoter, making an element that is now more active[87]. The duplication of IS*21* in R68 is therefore equivalent to the generation of the active intermediate stage in this process, increasing the rate of insertion into the chromosome.

Integration of self-transmissible elements into the chromosome does not necessarily depend on the homology provided by multiple and widely distributed DNA segments like IS elements. Integrative conjugative elements have site-specific integration systems that therefore show higher frequency establishment in species that contain a perfect target sequence[88]. In the absence of such a system when PHAGE or plasmids enter a cell in which they cannot replicate and there are no blocks of sequence homology, integration can occur by non-homologous recombination between short repeats of as little as 5 to 12 bp[64,89]. This seems to be a reverse of the illegitimate excision events that give rise to free circles of chromosomal DNA during the formation of SPECIALIZED TRANSDUCING PHAGE[90,91]. In a recent survey of genomes for the presence of sequences with high levels of identity to IncP-1 plasmid sequences, we found several examples with clusters of short segments with close to 100% identity, particularly to regions encoding transfer genes[92]. There was no evidence for transposable elements near the ends of these segments.

Mechanism of transfer and recipient specificity. Conjugative transfer is now recognized as a diverse collection of processes. The systems with greatest complexity are those encoded by large self-transmissible plasmids of Gram-negative bacteria that use a type IV secretion apparatus to produce a PILUS (the mating-pair formation apparatus). This structure mediates cell–cell

INSERTION SEQUENCE
A transposable DNA segment that normally only encodes the enzymes that mediate its own transposition and has no phenotypic marker.

TRANSPOSASE
The enzyme that promotes cutting the DNA at the ends of a transposable element and joining to the DNA molecule into which the element is to be inserted.

PHAGE
An abbreviation of bacteriophage — a virus that specifically infects bacteria.

SPECIALIZED TRANSDUCING PHAGE
A bacteriophage that integrates into a host-cell chromosome and then is excised again, bringing with it (as part of the phage genome) part of the host chromosome that can be transferred across to a new host.

PILUS
The proteinaceous fibre made from multiple subunits of a protein called pilin that mediates contact between donor and recipient bacteria prior to conjugative transfer.

contact to generate a junction between the bacteria and a pore through which the plasmid DNA and some donor-encoded proteins can be transported to the recipient[93]. For selected plasmid groups, there have been attempts to define what determines recipient specificity. One clear area of specificity is the interaction of the mating-pair formation apparatus (normally the pilus or its equivalent) with the lipopolysaccharides and outer-membrane proteins of the recipient cell surface[94,95]. However, some mating-pair formation apparatuses, for example, those encoded by the IncP and Ti plasmids, can form productive junctions with the surfaces of various cell types[96,97], including not only Gram-negative bacteria but also Gram-positive bacteria, yeast, plant and animal cells. Where it has been tested, the process seems to be the same[98]. Transfer efficiency of IncP-1 plasmids, as measured by the number of transfer events per donor present, can be dramatically affected by the identity of the donor — RK2 transfer between *E. coli* strains or from *E. coli* to *Pseudomonas putida* is less rapid than transfer between *P. putida* strains[99].

The conjugative transfer systems studied in Gram-positive bacteria are a more diverse set of processes than those of Gram-negative bacteria[100]. A well studied class of transfer apparatuses encoded by plasmids from the Gram-positive enterococci is only switched on in response to an appropriate recipient through production of PHEROMONES[101]. Most enterococcal strains produce multiple small, hydrophobic peptides that act as signals for interbacterial communication[102]. The result of activation of the transfer genes is the production of a membrane protein that promotes aggregation of the donors and recipients[103]. Each of the different transfer systems is activated by a different pheromone, but as the host bacteria could potentially activate themselves, the resident plasmid not only suppresses the production and release of the cognate molecule but also produces a peptide antagonist of the activating peptide. The result is a system that is tightly regulated, allowing transfer to only a limited group of bacterial species. However, other bacterial species also produce related peptides, so for example, *Staphylococcus aureus* will induce enterococci to enter the aggregative state, which might facilitate intergeneric spread of important traits such as vancomycin resistance[104,105]. Other enterococcal plasmids, like pIP501, have also been studied and found to encode promiscuous transfer systems[106].

Streptomyces plasmids are generally suggested to represent the other extreme from F, Ti and IncP-1 plasmids, as the well studied plasmids like pJI101 seem to rely on natural bridges between host HYPHAE, allowing the single plasmid-encoded transfer protein to promote movement from donor to recipient[107,108]. However, recent analyses of plasmid sequences[109–111] indicate that at least some *Streptomyces* plasmids encode multiple transfer proteins and might use a conjugative apparatus more similar to that of Gram-negative plasmids.

Many plasmids are not self-transmissible, but can nevertheless be mobilized from one bacterium to another in the presence of a self-transmissible plasmid. This normally involves specific interaction of MOBILIZATION proteins encoded by the plasmid and assembled at the transfer origin to form a RELAXASOME, with the mating-pair apparatus formed by the conjugative plasmid. Where it has been analysed, the specificity is determined by the coupling protein belonging to the VirD4/TraG family[112,113]. This interaction is therefore an important factor that can determine the frequency with which a given plasmid and its associated genes are involved in transfer.

Barriers to HGT between bacteria

Depending on the bacterial species involved and the gene-transfer mechanisms that are active, a number of processes limit the transfer, uptake and stabilization of foreign DNA molecules in bacteria. The limitations in the availability of adaptive DNA in the environment, in bacterial competence development, in DNA uptake specificity and in lack of DNA sequence similarity to facilitate integration of DNA into a replicating genetic unit have been discussed above. Whereas in transformation, it is the recipient that has the more active role that promotes HGT, in conjugative transfer it is the donor that seems to have the positive role and, by contrast, the recipient often has a negative role, limiting entry or establishment of the incoming DNA.

Surface exclusion as a barrier. The original observations with plasmid F in *E. coli* defined a phenomenon termed SURFACE EXCLUSION, which seems to create an effective barrier against conjugative transfer into bacterial cells that already carry the genes for a closely related transfer apparatus (reviewed in REF. 114). Detailed analysis showed that for F, surface exclusion works at two levels: TraT changes the outer surface of the cell and reduces its receptiveness to the F pilus about 10-fold, TraS sits in the inner membrane and prevents DNA entry by about 100-fold. Surface exclusion is widespread (but not universal) in conjugation systems of both Gram-negative[115–117] and Gram-positive elements[118]. However, studies of different F-like plasmids revealed that a large amount of interplasmid recombination has taken place[119]. Similarly, among the recently sequenced IncP-1 plasmids, one seems to be the product of recombination between two distinct parents[120]. Therefore, despite surface exclusion, plasmids can enter cells that carry a closely related element. For F, the surface-exclusion barrier is reduced in stationary phase in liquid cultures, in non-growing populations on agar plates and if bacteria are starved for carbon source[121]. This is consistent with the discovery that many plasmids promote biofilm formation, even in pure plasmid-positive cultures, by a mechanism that is dependent on their possession of a transfer apparatus[122]. This implies that under these conditions, surface exclusion is not preventing the adhesion promoted by the transfer pilus that is the first step in mating-pair formation[123]. It might be that surface exclusion has not evolved as a barrier to promote recombinational isolation of plasmids, but is more related to promoting the breakdown of mating pairs after gene transfer has taken place and release of the recipient to disperse the plasmid

PHEROMONE
A diffusible small molecule that can act as a chemical signal.

HYPHAE
The tube-like cellular growth associated with mycelial organisms.

MOBILIZATION
The process of a non-self-transmissible element being allowed to tranfer by the presence of a self-transmissible element.

RELAXASOME
The protein–DNA complex at the transfer origin that results in nicking of the DNA when the proteins are denatured chemically or cleaved proteolytically.

SURFACE EXCLUSION
The reduction of transfer frequency during conjugative transfer to recipients already carrying a related plasmid.

to new potential recipients. It might even be that the selection is on a totally different phenotype — in F, TraT also confers serum resistance[124]. The extent to which the conclusions from studies on F-like plasmids apply to other systems remains to be determined.

Restriction as a barrier. DNA that is recognized as foreign because it does not have the same sequence-specific chemical signatures can be broken into pieces by restriction endonucleases[125]. For small plasmids and individual genes, there is a significant chance that they might not contain some of the rarer restriction sequences and therefore avoid the effect of enzymic cleavage. In addition, the fact that DNA entering through conjugative transfer or natural transformation is single-stranded instead of double-stranded might provide some protection, and indeed, comparison with transformation frequencies of double-stranded DNA does confirm a greater ability to avoid destruction through the former mechanism[126]. Nevertheless, it is well established that the frequency of transconjugants can be reduced if the recipient has a restriction system to which the incoming plasmid is susceptible[127–129]. The broad-host-range IncP-1 plasmids seem to have adapted to the existence of such barriers by selection of variants that have lost most sites[130]. Introduction of a cloned DNA fragment with extra restriction sites into the plasmid increases the barrier, resulting in a reduced frequency of transfer, and therefore confirming that the barrier is roughly proportional to the number of target sites in the plasmid[128,130]. This is a practical problem for genetic manipulation of diverse bacteria, and solutions vary from using a non-methylating *E. coli* host in which the recipient recognizes methylated DNA[131] to cloning appropriate modifying enzymes into *E. coli* so the DNA is already protected on entry[127].

Plasmids carrying genes encoding products that are known to interfere with the action of type I restriction systems can enter restriction-positive hosts more efficiently than plasmids without such anti-restriction systems[132–134]. PARALOGUES of the *ardA* gene are found quite commonly in conjugative plasmids[135]. However, attempts to show interference with restriction by an ArdB paralogue in the IncP-1 plasmid RK2 were unsuccessful[136]. Surprisingly, the IncP-1β plasmids not only are less deficient in restriction sites than the IncP-1α plasmids, they also carry clusters of repeated sequences with type II restriction sites adjacent to each repeat, which might promote acquisition of mobile DNA and therefore favour dissemination of phenotypic markers[137].

Many plasmids carry restriction-modification systems[138] and this can potentially affect transfer to new hosts. If the host DNA is not modified, it should therefore be susceptible to degradation by the newly introduced restriction endonuclease. In practice, expression of restriction-modification systems in a virgin host proceeds in such a way that the modification component of the system is active first[139]. Alternatively, where the recipient restriction system recognizes methylated DNA as does DpnI, introduction of an appropriate methylase can result in death of most of the recipients[126].

Barriers to plasmid replication and establishment in a heterologous host. A key property that allows a plasmid to promote horizontal transfer of genes that have no ORTHOLOGUE in the recipient genome is its ability to replicate and therefore avoid the need for recombination into a replicon like the chromosome. Some plasmids have a broad host range, whereas others are more limited. The best-studied broad-host-range plasmids of Gram-negative bacteria belong to the IncP (IncP-1)[140] and IncQ (IncP-4)[141] groups of *E. coli* (and *Pseudomonas* spp.). The broad host range of IncQ plasmids like RSF1010 could be due to the plasmid encoding three replication proteins — an origin-activation protein, RepA; a HELICASE, RepB; and a primase, RepC[142]. As the plasmid does not have lagging-strand origins, normal replication requires recruitment of a DNA polymerase III as well as single-stranded DNA-binding proteins and any histone-like proteins that might be needed, all of which should have relatively little DNA specificity. Its segregational stability is largely due to its medium copy number[143]. The absence of LAGGING-STRAND SYNTHESIS, and therefore accumulation of single-stranded DNA-replication intermediates, puts constraints on the amount of DNA that these plasmids can acquire and therefore transfer horizontally without becoming recombinationally unstable. An IncG (IncP-6) plasmid, Rms149, the DNA sequence of which was determined recently[144], contains the IncQ-family *mob* genes but has acquired a different, more typical low-copy-number θ replicon with linked active partitioning genes, explaining its ability to have expanded to 57 kb through insertion of multiple transposable elements. The importance of lagging-strand synthesis for host range has been confirmed through studies on the rolling circle replication (RCR) plasmids of Gram-positive bacteria[145]. A host helicase that interacts with the Rep protein of such plasmids is also important and might be a crucial factor in efficiency of establishment in foreign hosts[146].

By contrast, IncP plasmids have just one *rep* gene, which encodes two related proteins, TrfA1 and TrfA2, that, with DnaA, provide flexible routes to recruitment of the host DnaB helicase, accommodating differences in dependence on accessory proteins such as DnaC[147,148]. The smaller Rep protein TrfA2 is sufficient for efficient replication in most species and combines with DnaA to activate the *oriV* region and recruit the host helicase. However, in *P. aeruginosa* DnaA is not needed and TrfA1 is adapted to achieve this recruitment[149,150]. In addition, the IncP-1 plasmids encode several auxiliary stable inheritance functions[137,151,152], which are coordinately regulated with the *trfA* gene in a complex regulon involving autogenously repressed strong promoters and which provide a buffer against variations in promoter strength in different species[99].

Studies on plasmids with narrower host ranges have revealed various limitations to successful replication. For plasmid F of *E. coli*, the barrier to replication in *Pseudomonas* species seems to be due to the inability of its replication protein, RepE, to effectively recruit DnaB to complete the activation of the replication

PARALOGUES
Homologous genes in the same organism that have evolved from a gene duplication and a subsequent divergence of function.

ORTHOLOGUES
Homologues that are related to each other through a speciation event.

HELICASE
Enzyme that unwinds DNA duplexes.

LAGGING-STRAND SYNTHESIS
Discontinuous synthesis on the strand running back from the replication fork, dependent on regular synthesis of primers by a primase and other primosome proteins.

origin after the initial RepE–DnaA–*ori* complex has been formed[153]. Although there seems to be a difficulty with expression of the *repE* gene, mutation to improve the promoter activity in *Pseudomonas* species does not overcome the barrier. It is not clear if mutants of F that can replicate in *Pseudomonas* species could arise easily. For pPS10, originally from *Pseudomonas syringae*, the reason for the plasmid's temperature-sensitive replication in *E. coli* is the lack of productive interactions between the plasmid-encoded Rep and DnaA, as indicated by mutations that allow replication at 37°C in *E. coli* mapping in either *rep* or *dnaA*[154,155]. Many IncP-9 plasmids are also lost at 37°C in *E. coli*[156], but our recent work indicates that the limitation seems to be due to inefficient *rep* expression in *E. coli*[157]. Although the *rep* promoter works in *E. coli*, it shows weak similarity to the σ^{70} consensus, and changing the –10 region or the –35 region so that they match the consensus allows the plasmid to replicate efficiently at 37°C (REF. 157). Therefore, if selective pressure were to exist, many plasmids could probably extend their host range by simple mutational changes. Indeed, plasmids of different host ranges can be found within the same plasmid family[158], indicating that host range adapts to the selective history the plasmid has experienced.

Conclusions

The main impact of HGT in bacterial evolution has been well established from recent large-scale bacterial DNA genome sequencing. Our mechanistic understanding of the processes that facilitate HGT events has come from the study of a few model organisms. Although several steps important to HGT have been identified and characterized, we are far from a thorough understanding of the environmental factors that promote or limit HGT events. Often, the impact of HGT events have been inferred from transfer frequencies observed in the laboratory. However, it is clear that frequency-based assessments do not adequately consider the environmental, temporal and spatial population dynamics of HGT events and their long-term impact. Therefore, even if the transfer frequency falls below a level that is detectable in the laboratory, ecologically important HGT events might still be occurring. It is important to note that laboratory-observed HGT events are rarely linked to relevant population-genetics parameters and temporospatial considerations. Bacterial genomes are in a constant state of flux, and any segment of DNA in a large bacterial population might have the opportunity to be horizontally transferred. However, only a minor proportion of the DNA transferred between species is likely to be maintained in the new host over generations. Many factors limit evolutionarily successful horizontal gene acquisitions: not only the mechanistic barriers to establishment, expression and function; but also temporal and spatial limitations to the dissemination of the descendants of transfer events that limit the spread of horizontally acquired genetic material in bacterial populations. On the other hand, the mechanistic barriers to horizontal gene acquisitions are under genetic, physiological and environmental modulation, so that under specific conditions, HGT might be favoured. A main research challenge is to understand how these modulations occur, so we can produce conditions to promote defined gene acquisitions when we are trying to manipulate a bacterial strain or a microbial community, or to prevent HGT in, for example, a hospital ward. The ecological impact of HGT events can only be fully appreciated when combining an improved mechanistic understanding of single HGT events with a population-dynamic consideration of the stochastic and selective conditions that determine the fate of HGT events in larger bacterial communities. Understanding selection in natural microbial habitats and relating these conditions to the genetic compositions of bacteria will be the main challenge in future microbial-ecology research. Further developments in methodology and theoretical and experimental approaches, discussed in the accompanying reviews (see the article by S.J. Sørenson *et al.* and the article by J.P. Gogarten and J.P. Townsend in this issue), will be needed to understand the roles of mobile elements and extracellular DNA in ecosystem dynamics and evolutionary processes.

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Competing interests statement

The authors declare no competing financial interests.

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