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STUDYING PLASMID HORIZONTAL TRANSFER *IN SITU*: A CRITICAL REVIEW

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Abstract | This review deals with the prospective, experimental documentation of horizontal gene transfer (HGT) and its role in real-time, local adaptation. We have focused on plasmids and their function as an accessory and/or adaptive gene pool. Studies of the extent of HGT in natural environments have identified certain hot spots, and many of these involve biofilms. Biofilms are uniquely suited for HGT, as they sustain high bacterial density and metabolic activity, even in the harshest environments. Single-cell detection of donor, recipient and transconjugant bacteria in various natural environments, combined with individual-based mathematical models, has provided a new platform for HGT studies.

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Mobile genetic elements can be exchanged promiscuously between a broad spectrum of bacteria and contribute to bacterial genome plasticity. As components of the horizontal gene pool, mobile genetic elements include insertion sequences, transposons, integrons, bacteriophages, genomic islands (such as PATHOGENICITY ISLANDS), plasmids and combinations of these elements. Driven in part by the accelerating pace of whole-genome sequencing, more and more evidence is emerging in support of significant gene shuffling by horizontal gene transfer (HGT) among members of the domains Bacteria, Archaea and Eukarya — including plants, fungi and human cells. In this review, we focus on plasmids and their function as part of an accessory and/or adaptive gene pool, and particularly the techniques used to analyse plasmid transfer. Cell TRANSFORMATION by free DNA and bacteriophage-mediated TRANSDUCTION, however, are also important mechanisms of HGT (BOX 1).

Plasmids are present in all branches of the bacterial 'tree of life' and have been found in all bacterial communities studied to date, including soil, and marine and clinical environments. However, only a limited amount of plasmid-sequence data are currently available[1]. Comparative analyses of the available data have revealed the complexity of plasmid genetics, the capacity of these elements to replicate autonomously in permissive bacterial cells and the presence of a unique set of genes that are clearly distinct from the genes that are typically found on bacterial chromosomes. The extent of the genetic diversity within the horizontal gene pool and its apparent distinction from the 'essential' core content present on bacterial chromosomes has encouraged speculation that plasmids represent a distinct genetic resource, yet the relationship between plasmids and their hosts is crucial for host ecology.

So, what are the roles of plasmids? Plasmids allow bacterial populations to 'sample' the horizontal gene pool for adaptive traits that might be advantageous for survival under local selective pressures, with antibiotic resistance and the ability to use novel carbon sources being simple examples of this. Plasmids also provide genetic variation, act as sources for recombination and, owing to their longer retention time in the cell, can allow faster gene fixation than either transformation or transduction, leading to a greater likelihood that the 'new' trait will persist.

PATHOGENICITY ISLANDS
A contiguous block of genes acquired by horizontal transfer in which at least a subset of the genes code for virulence factors.

TRANSFORMATION
The uptake and incorporation of exogenous, 'naked' DNA directly from the environment.

TRANSDUCTION
The horizontal transfer of DNA mediated by bacteriophage.

CONJUGATION
The transfer of DNA between bacterial cells after cell–cell contact. Conjugation is mediated by mobile genetic elements (usually plasmids or transposons) and is unidirectional and conservative (a copy of the DNA remains in the donor strain)

TYPE IV SECRETION SYSTEM
A syringe-like proteinaceous machinery that can transport bacterial protein or DNA effector molecules directly into a eukaryotic cell.

Plasmid conjugal transfer

Plasmids have a dominant role in the horizontal transfer of genetic information between bacteria and can transfer DNA between genera, phyla and even major domains[2,3] by a mechanism that is known as bacterial CONJUGATION. Conjugation involves direct cell-to-cell contact, mating-pair formation and DNA exchange mediated by conjugative pili. The genes responsible for the transfer of most transfer-proficient plasmids that have been isolated from natural environments have not been identified. However, sequencing of several plasmid-transfer regions has indicated that there is considerable diversity in both the genes and the operon structures involved[4].

The bacterial conjugation machinery is also referred to as a TYPE IV SECRETION SYSTEM (T4SS). T4SSs are functionally diverse and, in addition to the conjugal transfer of DNA, are also involved in DNA uptake and release in bacteria such as *Helicobacter pylori* and *Campylobacter jejuni*[5,6]. Furthermore, in several pathogenic bacteria, T4SSs are involved in the translocation of proteins into eukaryotic host cells during infection[7]. For detailed discussions on the mechanisms involved in T4SSs, readers are referred to recent reviews[7,8].

Despite the availability of molecular data, the precise biochemical mechanisms that regulate conjugative transfer remain elusive. Given that there are plasmids that cannot self-transfer but which, in the presence of transfer-proficient plasmids, can be mobilized from host to recipient or retrotransferred from the potential recipient back to the host[9], it is probable that several distinct transfer mechanisms have evolved.

Bacteria and their plasmids have co-evolved to provide key mechanisms for gene transfer and to ensure their own survival as components of the horizontal gene pool. The products of conjugational genes seem to have further ecological significance, as they promote cell-to-cell contact, which can facilitate biofilm formation[10]. As biofilms provide some protection against predation and fluctuations in local environmental conditions, it is arguable that the presence of conjugative pili, and not other conjugative functions or HGT itself, provides a direct selective advantage to the bacterial host[10].

Studying HGT: retrospective or prospective?

Two conceptually different approaches have been taken to assess the role of HGT in bacterial evolution. The first — a retrospective approach — consists of comparing the phylogenetic relationship between genes or proteins that have similar functions with the known phylogeny of the organisms from where these genes or proteins originated[11]. HGT is implicated as an evolutionary force if closely related genes (or gene sequences) are present in diverse and distantly related hosts. In bacteria, the presence of intergenomic variation can be interpreted as an illustration that HGT contributes profoundly to genetic diversity and evolution[12], and recent advances in DNA sequencing have facilitated this approach (see also the article by J.P. Gogarten & J.P. Townsend in this issue). However, it must be remembered that these analyses are constrained by the simple fact that one can only compare recombination events that are successful and integration events that have been conserved over the protracted evolution and selection of the bacterial chromosome as studied. Another line of retrospective evidence that supports the significance of HGT in bacterial evolution involves comparing genes that encode accessory catabolic pathways[13]. Many catabolic pathways seem to comprise various genetic modules that have assembled to give rise to new catabolic functions but which still retain evidence of their mobile nature by being defective transposons or flanked by insertion sequences[14,15].

The second approach — the prospective approach — is based on the direct experimental study of HGT as bacteria acquire, modify or lose known traits in defined systems. This approach is the focus of our review.

Studying the extent of HGT *in situ*

Direct evidence of extant *in situ* plasmid transfer in natural environments has typically been obtained by identifying plasmid-encoded phenotypes (for example, antibiotic resistance, heavy metal resistance or accessory catabolic pathways) following the introduction of donor strains. Such an approach relies on the co-introduction of a well marked recipient strain[16–18] or the emergence of identifiable phenotypes among indigenous bacterial populations[19,20].

Box 1 | Transformation and transduction

Horizontal gene transfer (HGT) in bacteria can occur by two other mechanisms besides conjugation. Transformation refers to the uptake of free DNA by a bacterial cell and its stable integration into the bacterial genome. Transduction involves the accidental packaging of cellular DNA into bacteriophage particles during replication. When the transduced phage infects another bacterial cell, the foreign cellular DNA then becomes part of the host genome.

Of the three mechanisms, transformation can be considered the only prokaryotic HGT process that does not rely on selfish genetic elements because the necessary genes reside on the chromosome. Approximately 90 transformable bacterial species have been identified, but not all are known to be competent for DNA uptake in the natural environment. Transformation involves the induction of competence, DNA binding, DNA fragmentation, DNA uptake and stable maintenance of the acquired DNA either by recombination or by recircularization of plasmid DNA. In liquid culture, the state of competency is growth-phase-dependent in most organisms studied. By contrast, biofilms of *Acinetobacter* spp. grown in flow cells are continually competent and some bacteria are always capable of taking up DNA, such as *Thermus thermophilus* and *Helicobacter pylori*. Uptake machineries are similar in Gram-positive and Gram-negative bacteria and include components of type IV pili and TYPE II PROTEIN-EXPORT SYSTEMS. Only some bacteria, including *Bacillus subtilis*, *Streptococcus pneumoniae* and *Acinetobacter* spp., take up DNA from unrelated species. Others recognize related DNA by specific nucleotide-sequence tags that are overrepresented in their own genomic DNA. For the stable recircularization of plasmid DNA, two single strands with partially complementary sequences must enter the same cell to allow efficient repair by host enzymes.

Transduction is a specific HGT process, as bacteriophages have a limited host range. Yet bacteriophages are important gene-transfer vehicles, owing to their great abundance and the ability of temperate bacteriophages to insert themselves into chromosomes as a prophage without causing cell lysis, therefore altering the genetic content of their hosts. Many bacteria contain several prophages, which can also encode virulence genes, and the full contribution of transduction to lateral gene transfer, including transfer of plasmid-borne genes, will only be realized once a sufficient number of phage genomes have been sequenced.

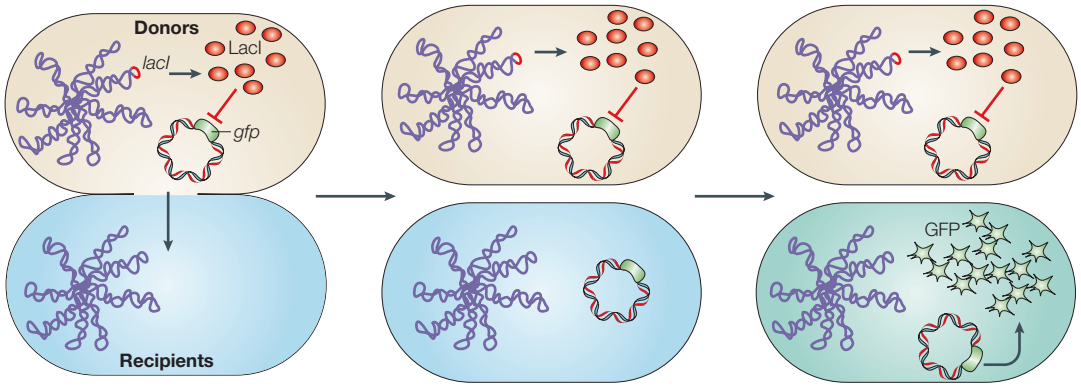

Figure 1 | **A schematic outline of the transfer reporter-gene approach.** The donor cell contains a conjugative plasmid tagged with the green fluorescent protein (GFP) gene (*gfp*) downstream from a LacI repressible promoter. The donor chromosome encodes LacI, which represses the expression of GFP. During conjugation, the plasmid is transferred from the donor cells to the recipients, which become transconjugants. Expression of *gfp* is not repressed in the transconjugant cells, and these cells consequently fluoresce green. In contrast to previous detection methods, which rely on successful cultivation of transconjugants on selective media, the transfer reporter-gene approach only requires that recipient cells can express the *gfp* gene cassette. Image courtesy of G. Oregaard, University of Copenhagen, Denmark.

The conjugal transfer of plasmids occurs in various environments[21]. However, prospective studies have identified certain environmental hot spots for gene transfer and environmental factors that can influence the frequency of conjugal gene transfer. In terrestrial environments, for example, geophysicochemical factors such as soil type[22], soil moisture[23], temperature[23], pH[24] and water movement[25] have all been identified as factors that influence the frequency of plasmid transfer. Biological factors, including the presence of protozoa, earthworms or fungi[26–28], and abiotic factors such as nutrient amendments[29] also influence HGT in soils. Although some studies have suggested stimulatory effects of stresses, such as metal stress, on conjugation rates[16], the available data are ambiguous, as the survival of TRANSCONJUGANTS might be enhanced by the presence of the stress if resistance or tolerance to this stress is encoded by the transferred plasmid[18,30,31]. Inevitably, environmental factors also influence bacterial activity and, ultimately, the density of the donor and recipient populations. Indirect effects on population size and relative densities might therefore have had a greater influence on the recorded plasmid-transfer frequency in most of these studies than did the direct effects on plasmid transfer.

As there is no consensus concerning the best system to report the extent of plasmid transfer, it is difficult to compare results from different studies. Clearly, a standardized reporting method for plasmid-transfer efficiency must be found. In theory, plasmid-transfer efficiency should be reported as the number of transfer events per donor–recipient encounters. Such a ratio would allow true comparisons of the transfer efficiencies of different plasmids or in different environmental settings. These kind of data could in the future be obtained by online single-cell detection. Here, we will use a simple transconjugant to donor ratio (T/D), as this ratio, although low in descriptive power, can be extracted from most studies. Generally, the efficiency of plasmid transfer in bulk environments such as bulk water and bulk soil is low (T/D typically $<10^{-5}$) and in many cases transfer could only be detected after nutrient enrichment[29]. This is in contrast to hot spots of bacterial metabolic activity and HGT, including the RHIZOSPHERE and PHYLLOPLANE of plants and other biofilm-supporting environments, where T/D ratios can typically be as great as 10^{-3} or even 10^{-1} for indigenous as well as introduced plasmids[3,32].

Methodological problems

Three important methodological problems must be overcome when attempting to assess the extent of HGT by plasmids in any given environment. First, traditional methods for detecting gene transfer that rely on culturing do not distinguish between an increased number of transfer events and post-transfer selection (clonal expansion of the recipient population). Second, traditional methods produce population-averaged measures of gene transfer, which generally do not provide an insight into the spatial extent of HGT, especially within microcolonies and biofilms, which are highly variable, non-homogeneous environments. Third, traditional approaches invariably rely on cultivation for the enumeration of donor, recipient and transconjugant populations.

It is well known that the culturable fraction of bacteria is often <1% of the total number of bacteria determined by direct counts[33,34], and that the community structure (that is, the relative proportions of different subgroups present) can change drastically when enriched with nutrients[35]. Culturing might also be biased owing to the so-called VIABLE BUT NON-CULTURABLE state, which some bacteria have been reported to enter[36]. The gene-transfer studies cited in the previous section were based on cultivation techniques. Therefore, the overall extent of the HGT of plasmids in the environment examined might have been underestimated.

TYPE II PROTEIN-EXPORT SYSTEMS
The type II protein-export system or secreton allows the energy-dependent secretion of specific proteins from the periplasm of Gram-negative bacteria.

TRANSCONJUGANTS
Bacterial recipient cells that have recieved a plasmid by the process of conjugation as indicated by molecular, phenotypic or microscopic analysis.

RHIZOSPHERE
The zone directly surrounding the roots of plants.

PHYLLOPLANE
The micro-environment on the leaf surface of plants.

VIABLE BUT NON-CULTURABLE
A hypothesis that assumes a physiological state of normally culturable bacteria in which they no longer grow on conventional media but remain intact and retain viability.

New approaches to detect plasmid transfer

Reporter-gene technology. New methods have been suggested for studying plasmid and gene transfer without the need for culturing. Reporter-gene technology, for instance, removes the need for cultivation and allows estimation of the total number of cells (culturable and 'non-culturable') in which the reporter gene is expressed. This technology also allows the non-disruptive *in situ* observation of individual cells (see below).

Various reporter genes have been used to examine HGT, including the luciferase genes *luxAB*[37] and *luc*[37,38], the β-galactosidase gene *lacZ*[39] and genes encoding fluorescent proteins such as green fluorescent protein (GFP)[40–42]. Only fluorescence genes have been used for the *in situ* detection of HGT in natural environments, as no substrate has to be added to detect the product of the expressed reporter gene. Recently, a dual-labelling technique has been developed, in which the chromosome of either the donor or the recipient is tagged with a red fluorescence gene (*rfp* or *dsRed*) and the plasmid is labelled with *gfp*[43–45]. In this case, localization of the donor and recipient cells as well the transconjugants is possible without affecting cell viability.

The expression of a *gfp* reporter gene was used in a study of conjugation on bean leaves by inserting the *gfp* gene downstream of a modified *lac* promoter on the test plasmid. The *gfp* gene was not expressed by the donor cells owing to a chromosomally encoded repressor (*lacI*^{q}) (FIG. 1). When the plasmid was transferred to indigenous bacteria on bean leaves, *gfp* was derepressed and green fluorescent transconjugant cells could be detected by EPIFLUORESCENCE MICROSCOPY[40]. In the marine environment, Dahlberg *et al.* used a similar approach to study conjugal gene transfer[42]. One of the advantages of using GFP or other fluorescent markers is that flow cytometry can be used for rapid enumeration and analysis of single bacterial cells, and >1,000 cells can be analysed per second[46]. Recently, a novel flow-cytometry-based method to rapidly determine the occurrence of gene transfer in a culture-independent manner was described[47]. The approach was used to study the efficiency of transfer of a GFP-marked conjugative IncP plasmid (pKJK10) from an *Escherichia coli* donor to indigenous freshwater bacteria in MICROCOSM EXPERIMENTS. Individual transconjugant freshwater bacteria were readily detected in two independent experiments. The efficiency of transfer (T/D) was >10^{-2}, and the number of transconjugants detected was 20–100-fold higher than counts based on cultivation (G. Oregaard & S.J.S., unpublished observations). Similar differences were found when the transfer efficiency of plasmid pKJK10 to indigenous rhizosphere bacteria was studied using the same technique (S. Musovic *et al.*, unpublished observations). In biofilms, the frequency of conjugative transfer of the IncP mobilizable (mob^{+}tra^{-}) plasmid pRK415 from *E. coli* to *Ralstonia metallidurans* was 1,000-fold higher when measured by quantitative microscopy than by standard plating methods[48]. Furthermore, many of the original transconjugants lost the plasmid during the first cell division despite the presence of selective

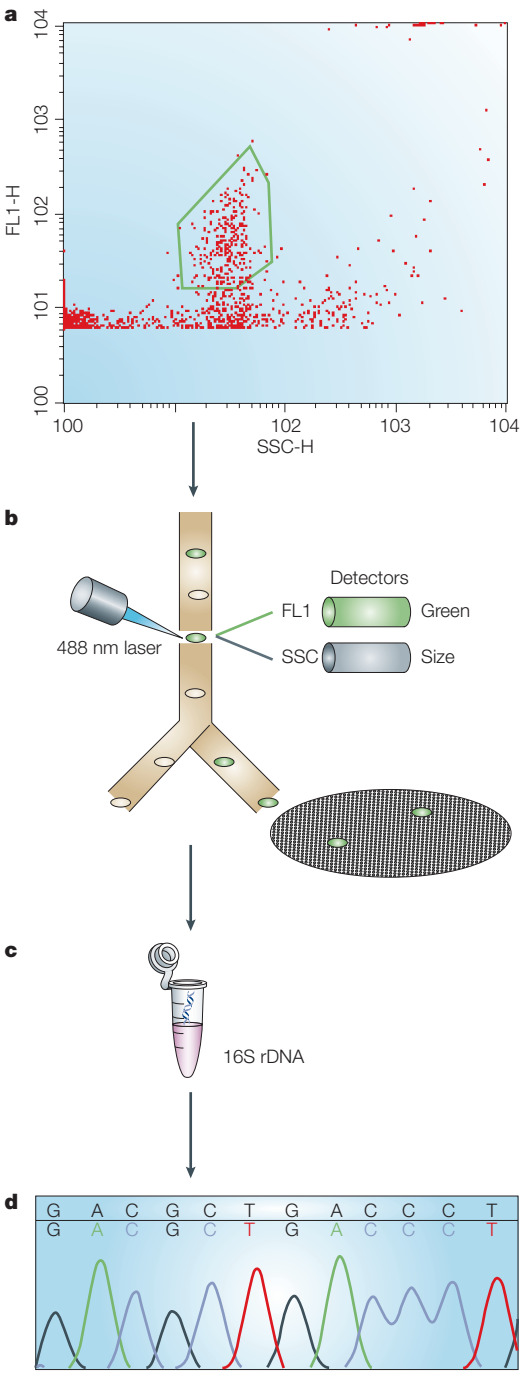

Figure 2 | **Detection of the host range of horizontal gene transfer (HGT) without cultivation.** HGT to indigenous bacteria in natural environments can be detected without cultivation using transfer reporter-gene technology as outlined in FIG. 1. Green fluorescing cells can be detected and enumerated by flow cytometry (**a**). The graph shows a dot-plot of green fluorescence (FL1) versus side scatter (SSC). The transconjugant cells defined by the green gate can be isolated by fluorescent activated cell sorting (FACS) onto membrane filters (**b**). Subsequently, DNA from the transconjugant community can be isolated from all sorted cells. 16S rDNA can be amplified by specific PCR reactions (**c**), and 16S clone libraries can be produced. Information on the phylogeny of the indigenous transconjugant bacteria can be obtained by sequencing the clones (**d**).

EPIFLUORESCENCE MICROSCOPY
A form of light microscopy that involves the detection of primary fluorescence or of specimens stained with fluorescent dyes.

MICROCOSM EXPERIMENTS
Experiments in scaled-down replicas of natural environments.

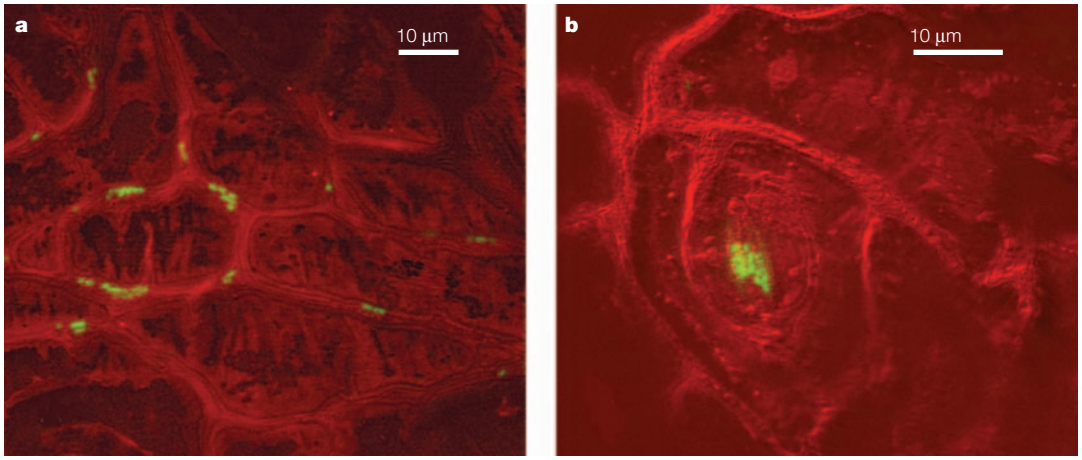

Figure 3 | **Horizontal gene transfer (HGT) in the phyllosphere.** Confocal scanning laser micrographs showing green fluorescent transconjugant cells in the interstices of epidermal cells (**a**) and inside a stoma (**b**) of a bean leaf. The donor (*Pseudomonas putida* plasmid KT2442) harboured a derivative of the TOL plasmid, which had the green fluorescent protein (*gfp*) gene inserted downstream of a *lac* promoter. A chromosomal insertion of *lacI*^{q} prevented expression of the *gfp* gene in donor cells. Transconjugants could therefore be visualized *in situ* as green fluorescent cells. Sterile bean seedlings were inoculated with donors and recipients (*P. putida* KT2440) at densities of approximately 10^5 cells per cm^2. Reproduced with permission from REF. 40 © (1998) American Society for Microbiology .

pressure. Together, these studies show the value of, and the need for more, culture-independent methods to determine the true outcome of HGT and plasmid maintenance in natural environments. However, it should be noted that not all transconjugant bacteria can be detected by reporter-gene technology. Some transconjugants might not efficiently transcribe the reporter gene, and codon usage can also hinder translation of reporter-gene mRNA in certain bacteria.

Using reporter genes to analyse plasmid host range. The host range of a plasmid depends both on successful transfer of the plasmid from the donor to the recipient cell and on the stable replication and maintenance of the transferred plasmid in the transconjugant. Some plasmids (for example, F factor) have a narrow host range, whereas others are transmitted more broadly (for example, RP4 (REF. 4)). Although shuttle vectors have been constructed that can replicate in both Gram-negative and Gram-positive bacteria[49], conjugal transfer of natural plasmids is believed to be confined within these groups.

Only a few studies have investigated the host range of plasmids among indigenous bacteria in natural environments. In most of these studies, *Pseudomonas* spp. and related bacteria that belong to the γ-subgroup of the Proteobacteria have been identified as the most frequent recipients for IncP plasmid transfer among the indigenous bacteria[29,50]. All these host-range studies relied on cultivation of transconjugant bacteria, an approach that fails to detect most naturally occurring bacterial cells.

By combining the culture-independent *gfp*-based gene-transfer detection approach with fluorescence-activated cell sorting (FACS), green fluorescing indigenous transconjugant bacteria can be isolated (FIG. 2). The transconjugant cells can subsequently be characterized by cloning and sequencing of 16S rDNA. A recent study used this approach to assess the host range of a natural barley rhizosphere plasmid (the *gfp*-labelled IncP plasmid pKJK10 described above) in indigenous rhizosphere bacteria. The plasmid was transferred from a *Pseudomonas putida* donor to indigenous bacteria belonging to the α-, β- and γ-subgroups of Proteobacteria. Surprisingly, one-third of all transconjugants were Gram-positive bacteria with high sequence similarity to *Arthrobacter* spp. (S. Musovic *et al.*, unpublished observations). Such reports underline the necessity to include the 'non-culturable' bacteria in this type of study. Although many more studies are needed before any general conclusions can be drawn, these results indicate that conjugal gene transfer could be far more extensive and common than was previously thought.

***Detecting HGT hot spots* in situ.** As previously stated, several environments have been identified as HGT hot spots. The term 'hot spot' is generally used in the literature to describe any environment in which the total number of gene-transfer events is high and observable. High-resolution *in situ* studies have provided new insights into the occurrence and efficiency of HGT within these environments. In the PHYTOSPHERE, for example, elevated transfer frequencies have generally been attributed to plant exudates stimulating bacterial metabolic activity[29,32]. However, *in situ* techniques have shown that HGT primarily occurs in niches characterized by high bacterial densities, such as in the junctures between epidermal cells, in sub-stomatal cavities[40] and on the HYPOCOTYL[43] (FIG. 3). This indicates that the availability of nutrients does not affect HGT through stimulation of bacterial metabolic activity but instead that the available nutrients allow the bacteria to form localized dense aggregates, or microcolonies, where the likelihood of cell-to-cell contact is high.

PHYTOSPHERE
All plant-associated environments, for example, rhizosphere or phylloplane.

HYPOCOTYL
The area of the plant-embryo axis below the area where cotyledons are attached that forms the primary root of a seedling.

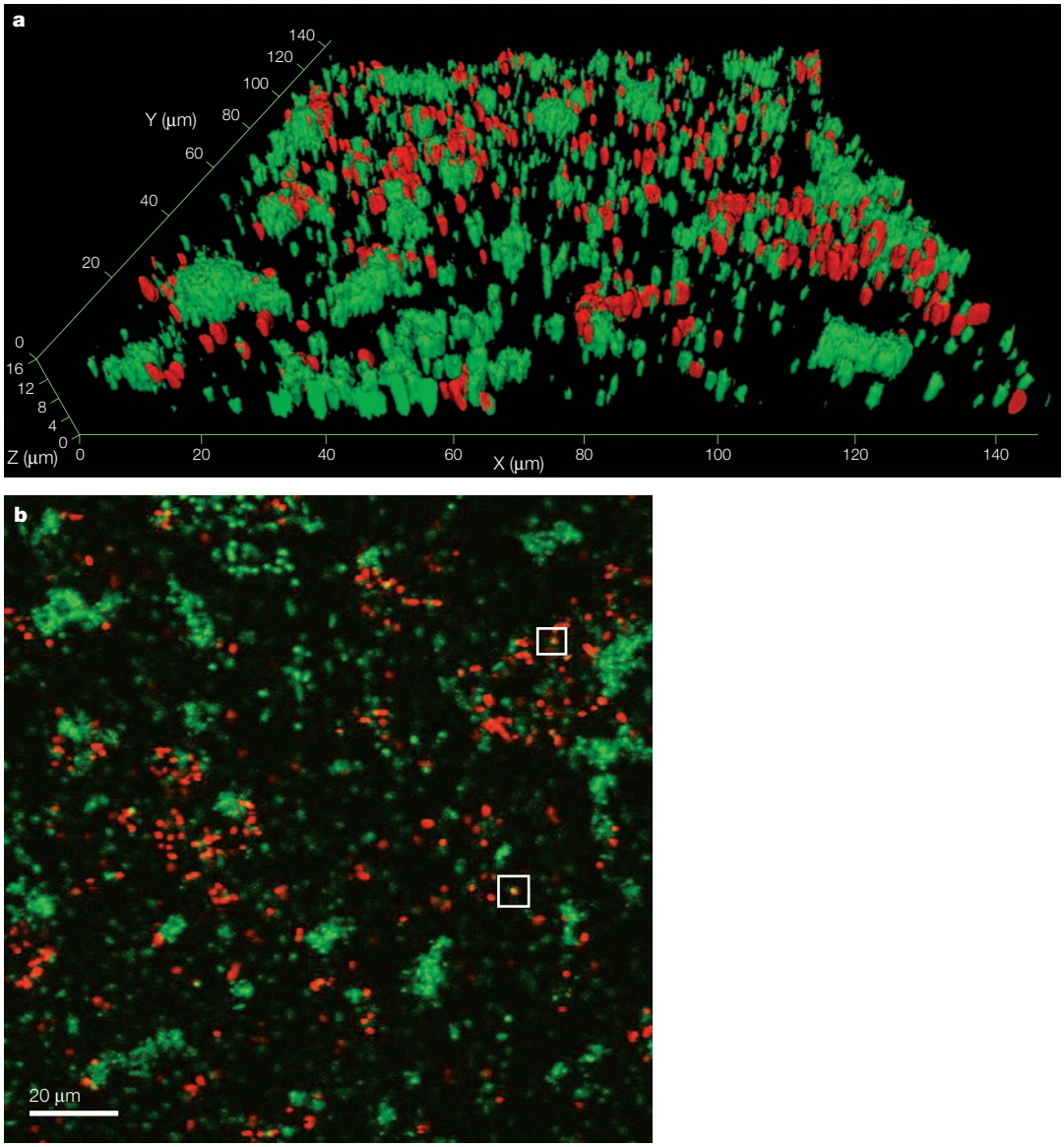

Figure 4 | **Horizontal gene transfer in biofilms. a** | Three-dimensional surface rendering of a *Delftia acidovorans* ATCC 15668 biofilm viewed with a LSM 520 Zeiss confocal laser scanning microscope. The biofilm was grown for 3 days under continuous flow conditions before *Pseudomonas putida* UWC3 (pWDL7::*rfp*) plasmid donor cells were added. pWDL7 is a self-transferable IncP1-b plasmid of ~80 kb that encodes part of the pathway to degrade 3-chloroaniline and 3,4-dichloroaniline as sole carbon and nitrogen sources[94]. Transfer was observed after the biofilm had been grown for an additional 5 days with 100 ppm 3-chloroaniline as sole carbon source. The biofilm underwent fluorescence *in situ* hybridization with the 5′-Cy5-labelled Bet42a probe specific for the β-proteobacteria, which hybridized with *D. acidovorans* ATCC 15668. Red cells are expressing red fluorescent protein (RFP) encoded on the plasmid pWDL7, whereas green cells are hybridized with Bet42a. Transconjugants are not visible in this view. Image courtesy of R. GrayMerod, University of California, USA. **b** | Plane view of the biofilm at a distance of 5 μm from the substratum. Transconjugants (shown in yellow as outlined by white box) are *D. acidovorans* ATCC 15668 cells that have acquired pWDL7, and therefore display overlap of the RFP fluorescence signal from pWDL7 and Cy5 after hybridization with Bet42a. Image courtesy of H. McCaslin, University of California, USA.

Biofilms. Biofilms exist at solid–liquid, solid–gas, liquid–liquid or liquid–gas interfaces. They consist of microorganisms, their excreted metabolic products (known as extracellular polymeric substances), various organic and inorganic particles, and water. Biofilms are ideal habitats for the development of diverse microbial communities, as the formation of chemical gradients facilitates the spatial heterogeneity of cell populations[51]. They occur in aquatic systems[52], including wastewater, sludge[53–55] and the EPILITHON[56]; in the terrestrial environment[57], particularly the phylloplane[40] and the rhizosphere[32,58]; and in the intestinal, urogenital and respiratory tracts[59–61]. There is no accepted definition as to the minimum thickness or surface area that must be reached before an agglomeration of microbial cells can be termed a biofilm.

EPILITHON
Biofilms attached to rocks in rivers and streams.

Biofilms are uniquely suited for HGT because they sustain high bacterial density and metabolic activity even in the harshest environments[62]. They provide a stable physical environment for cell–cell contact and extracellular DNA itself can contribute to the maintenance of biofilm structure (for review see REF. 63).

Insights into the extent and significance of HGT in biofilms are being gained from methodological advances that combine fluorescently tagged plasmids and bacterial strains with confocal laser scanning microscopy and quantitative image analysis[44,63,64]. Using this approach, information concerning the localization and distribution of transconjugants within a biofilm has been obtained (FIG. 4). Christensen *et al.* studied transfer of the *P. putida* TOL plasmid in gnotobiotic flow-chamber biofilm communities[65]. Transconjugants were preferentially formed on top of recipient microcolonies, whereas invasive transfer from new transconjugants to the other available recipients in the microcolony was not observed. The transconjugants had a selective growth advantage, and online monitoring of transconjugant proliferation indicated that the plasmid was primarily transferred vertically after a few initial horizontal-transfer events. Similar observations were made by Haagensen *et al.*[44] Again, transconjugants were established on top of recipient microcolonies but did not seem to spread inside the biofilm. The frequency of transfer was dependent on access and the establishment of cell-to-cell contact between donor and recipient. As new transconjugants rarely become donors, additional factors must regulate transfer. Perhaps new cell-to-cell contacts are necessary to trigger mating-pair formation, which, owing to inefficient mixing within the biofilm, could be limiting[40,63].

Clearly, the spatial structure or architecture of the biofilm has a decisive role in gene transfer. HGT by conjugation can be analysed by considering the physical environment encountered by donor cells travelling inside a biofilm matrix[51]. Transconjugants were found deep inside biofilms grown in flow cells, showing the ability of donor cells to penetrate beyond superficial surface layers (M. Hausner *et al.*, unpublished observations). Cell clusters occurring throughout a *Sphingomonas* sp. biofilm supported conjugative gene transfer. Three-dimensional image analysis revealed that more transconjugant cells were detected in the interior part of cell clusters. We hypothesize that open channels and pores enable more-frequent cell collisions, leading to rapid spread of plasmid-borne genes by conjugative gene transfer.

An important drawback to the use of GFP-based reporter plasmids for *in situ* studies is that the fluorescence of GFP can be impaired by some environmental conditions, such as high salt, low pH and lack of oxygen, which is particularly relevant in dense biofilm structures. Furthermore, the expression of GFP in metabolically inactive or weakly active cells is often weak or absent and can therefore not easily be distinguished from background fluorescence.

Although limited in number, the *in situ* studies carried out to date have provided important information on microscale HGT, and future studies should continue to take advantage of transfer reporter genes. However, instead of being used to endlessly study one environment after another, *in situ* techniques should be coupled with efforts to model HGT in attached communities by facilitating comparisons between predicted and experimental observations, and assist in establishing predictive frameworks.

Measuring plasmid stability

What is the fate of conjugational plasmids in natural environments? Plasmids can of course be maintained in a bacterial host in environments where plasmid-encoded traits provide a selective advantage, for example, antibiotic resistance in clinical environments. However, the fate of plasmids under non-selective conditions is perhaps more intriguing. Once established, natural plasmids seem to be highly stable and are not easily lost from strains grown under laboratory conditions. It therefore seems logical that plasmids are stably inherited and vertically distributed. If so, the plasmid-loss parameter could be omitted from the mathematical models that are used to make predictions relating to plasmid distribution in natural habitats. However, if plasmids are not lost, all bacterial cells in natural environments should, over time, accumulate one plasmid from each plasmid incompatibility group. Alternatively, the metabolic burden of the plasmid on the host is generally sufficient under neutral selective pressure to provide plasmid-free cells with a growth advantage. Therefore, an equilibrium probably exists between plasmid transfer, plasmid loss and bacterial growth rates.

Plasmids are present at one or more copies per cell. This provides an additional survival mechanism, as the probability that a plasmid-free daughter cell will arise by random segregation is directly related to the plasmid copy number ($P_0 = 2^{1-n}$, where P is the probability and n is the plasmid copy number)[2]. Theoretically, for plasmid pBR322, which is present at approximately 21 copies per cell, only one cell in 1,000,000 would be plasmid free; at 31 copies per cell the probability drops to one in 1,000,000,000.

Large plasmids are always maintained in low copy numbers in the cell, perhaps owing to the metabolic burden. To facilitate plasmid maintenance, the genes involved in plasmid stability are inherent to large conjugative plasmids[4]. Stability-enhancing proteins underpin the different types of mechanisms identified: post-segregational cell killing (*psk*) of plasmid-free cells[66,67], stable partitioning (*par*) of plasmids into daughter cells[68] and multimer-resolution systems (*mrs*)[69] (BOX 2). Similar mechanisms are also present on bacterial chromosomes, ensuring that each daughter cell has a copy of the genome during cell division.

On the other hand, conjugation itself could prove to be an important contributor to plasmid maintenance. If a plasmid is lost from an individual cell in a biofilm or a microcolony, the probability of encountering a donor

of the same plasmid is thought to be high, although it is uncertain whether effective transfer will result. Owing to methodological limitations, plasmid stability and survival in natural, non-clinical environments have only been investigated in a limited number of cases. A few studies have examined the survival and loss of plasmids in natural lake water, soils and the phytosphere[70–74]. Although plasmid loss was detected in some of these experiments, the factors that influence segregation and loss under natural conditions remain elusive. However, it is clear that segregation rates are significantly reduced when plasmid and host/donor bacteria are indigenous to the study habitat, indicating local adaptation.

Methods for detecting plasmid loss tend to rely on replica plating, selective markers such as antibiotic or heavy-metal resistance, and PCR detection[75] of genes inherent to the plasmids investigated. These methods have limitations for the study of natural populations, especially where segregation rates are low. In an attempt to increase sensitivity, Bahl *et al.* monitored plasmid loss in real time[76]. The production of GFP was repressed in plasmid-containing cells, therefore, cells that had lost their plasmids fluoresced green. By combining GFP reporter genes and flow cytometry it was possible to estimate the loss of different plasmids from bacterial hosts under laboratory conditions. This technique should allow future environmental studies of plasmid loss with high precision. Such future studies will be important for mathematical modelling of plasmid transfer and maintenance in natural environments.

Mathematical modelling of HGT

It is thought that most HGT takes place at interfaces supporting microcolonies and biofilms, yet most mathematical models of HGT have been developed for homogeneous mixed suspensions. Many studies have reported the incidence of extant transfer of plasmids in laboratory systems and open natural systems. However, quantitative treatment of this phenomenon has been largely limited to computing transfer frequencies, typically defined as the number of transconjugants over the number of donor or recipient cells[42,48], or the number of transconjugants per recipient cell per hour[48]. The weakness of these approaches is their limited predictive power, that is, plasmid transfer in another setting cannot be predicted or interpreted. Therefore, there is a need to develop and validate predictive mathematical models for HGT in surface-attached microbial communities.

A notable departure from the qualitative studies on plasmid transfer are the mass-action-based models, developed in the late 1970s by Levin and co-workers to describe the transfer of antibiotic-resistance-encoding plasmids between Enterobacteriaceae[77–80]. In these models, plasmid transfer is assumed to depend on a random encounter or 'collision' between donor (or transconjugant) and recipient cells. The formation of new transconjugants over a certain period of time is therefore directly related to the density of donors and recipients through a proportionality constant, termed the intrinsic plasmid-transfer-rate coefficient[78].

The model developed by Levin *et al.* comprises several differential equations that describe the concentration changes of donor, recipient and transconjugant cell types as well as the limiting growth substrate, and the model recognizes the processes of cell growth, plasmid transfer and plasmid loss[78,81,82]. This model has been used successfully to describe plasmid transfer in well-mixed suspended systems[60,83,84]. It has been shown that the physiological state and exogenous

Box 2 | **Plasmid stability systems**

The mechanisms that are involved in plasmid maintenance include post-segregational cell killing of plasmid-free cells, stable partitioning of plasmids into daughter cells and multimer resolution systems.

Post-segregational cell killing (*psk*) is a toxin–antitoxin mechanism that kills off plasmid-free bacteria after segregation. Well characterized *psk* systems include the *hok–sok* locus of *Escherichia coli* plasmid R1 and the *ccdB–ccdA* locus from the *E. coli* F plasmid. In the presence of the plasmid, both the stable toxin (in these systems, encoded by *hok* and *ccdB*) and the unstable antitoxin (encoded by *sok* and *ccdA*) are produced in cells. If a cell loses the plasmid, the unstable antitoxin is degraded and the toxin causes cell death.

Post-segregational cell-killing genes have been exploited for commercial use in systems such as cloning vectors that use the *ccdB* gene to ensure that only plasmids disrupted with a cloned insert are propagated. Similar suicide genes are also found on bacterial chromosomes, for example, *E. coli* K12, but their chromosomal roles are not clear.

The stable partitioning (*par*) system consists of three determinants in an active partition process. Typically, two *trans*-acting loci *parA* and *parB*, encoding plasmid proteins ParA and ParB, and a *cis*-acting site such as *parS* participate in the segregation process. ParA, an ATPase, binds to ParB, which in turn binds to the centromere-like *parS* sequence on the plasmid. These nucleoprotein complexes then assemble in the cells at separate locations with respect to the division plane, not unlike mitosis in eukaryotes. The plasmid-borne *par* systems resemble the chromosomal *par* systems.

A third mechanism involved in stable plasmid maintenance is the multimer resolution system (*mrs*). When plasmids are replicated, multimers or catenanes are formed. If unresolved, some daughter cells will receive more than their share of plasmids and thereby increase the chance of one of the daughter cells becoming plasmid-free. Therefore, almost all plasmids and chromosomes have genes that encode enzymes with resolvase activity. One example is the *mrs* system encoded by the *parCBA* operon of plasmid R2K. Here, the *parA* gene encodes a resolvase, which acts on the plasmid resolution site (*res*) to resolve plasmid multimers. This ensures that plasmids are separated from each other to become separate entities.

carbon availability mainly have an impact on the intrinsic plasmid-transfer-rate coefficient[60,82,85], whereas large effects of plasmid transfer by donor type have been observed with both *E. coli* and *Pseudomonas* spp.[86,83,84] Despite the tremendous contribution made by the models developed by Levin and co-workers, these models do not take into account factors such as a lag phase in the ability of a newly formed transconjugant to act as a proficient donor, or donor 'saturation' in cases of excessive numbers of recipients.

The currently available mathematical models for plasmid dynamics in surface-attached communities are, unfortunately, of limited value, as they either assume complete mixing (artificially attainable by initiating with confluent and high cell densities[87]), or are dependent on initially separate parental colony perimeters coalescing, with instantaneous plasmid transfer to an entire recipient community[88]. The assumption of a completely mixed, homogeneous environment with population-averaged behaviour is not applicable to track plasmid dynamics on surfaces where the distribution of cells is patchy[60,65,89]. Normander *et al.* studied plasmid transfer on the phylloplane of beans and showed that transconjugants were almost exclusively formed in the junctures between the epidermal cells and in the STOMATA[40]. Current models can neither describe nor predict such patterns of plasmid spread. Therefore, there is a dire need for a new generation of mechanistically valid plasmid dynamic models that reflect the environmental conditions in surface-attached microbial communities.

Given that spatial structure is paramount when species interact, the development of individual-based models (IbMs)[90,91] is an interesting strategy. The use of IbMs is a bottom-up approach that attempts to model a population or community by describing the actions and properties of the individuals that comprise this population or community[92]. In the IbM BacSim, developed by Kreft *et al.*[92,93], bacterial cells are modelled as discrete individuals of spherical shape that move in continuous space, instead of on a grid as in cellular automaton-based models. The IbM approach might be more appropriate for modelling genetic transfer by conjugation, as this is a discrete process between two individual cells that form a mating pair. This model also takes into account the changes that occur during the transfer process, for example, the different properties of the potential recipients when compared with transconjugants. The development of individual-based conjugal models will facilitate the interpretation of experimental observations on the role of plasmid transfer by providing a quantitative and predictive framework for understanding bacterial community response and adaptation.

Future challenges

More genetic-sequence information on the components of the horizontal gene pool is required. Targeted international action to gather more data on natural plasmid diversity in relation to host chromosome analysis should help to define what plasmids are, and also determine whether plasmids have lineages that approximate or are distinct from their bacterial hosts. Such knowledge will shed light on how bacteria exchange genes and adapt to their environment. For example, is the assumption that bacteria partition traits between plasmids and chromosomes correct? More sequence and biological data on the full spectrum of plasmids are required to answer this question.

It is well known that plasmids carry various traits that facilitate, or are essential for, adaptation to variations in habitat conditions, but more detailed functional studies of plasmid-encoded genes are required. We hold that such detailed analyses of the horizontal gene pool will identify novel and potentially key functions that could not be discovered by the study of bacterial isolates alone. The mobility of plasmids and their genes provides a strong argument that highly specialized genes are only present on mobile genetic elements. This mechanism implies that the vectors of these genes — the plasmids — ensure a fitness advantage, and that the plasmids themselves are both selected for and retained. In addition, they can transfer to new hosts where both plasmid and host find advantage in the partnership. The ecology of plasmids is still poorly understood and we know little of their distribution and diversity. Are they in fact ubiquitous? Is transfer in the natural environment as common as recent non-cultivation-dependent experiments imply, or are these studies peculiar to only certain habitats? The true rates of transfer in the natural environment have not yet been determined.

Do plasmids survive by stable maintenance mechanisms or by providing advantageous traits to the host? Perhaps plasmids simply transfer whenever the opportunity arises, that is, when the relative densities allow the direct cell–cell contact that precedes mating-pair formation. Alternatively, they might use transfer to escape less-fit hosts or opportunistically transfer to permissive recipients after the clonal expansion of the current host, therefore benefiting from selective advantage. Can plasmids regulate their host to induce a survival mechanism under adverse conditions? At present, we simply do not know enough about their basic biology, let alone molecular biology and genetics, to answer these questions and provide a more complete description of the role of plasmids in bacterial ecology and evolution.

Recent advances in fluorometric detection of single bacterial cells by fluorescent microscopy and FACS have provided the technology that is needed to study HGT directly *in situ*. New culture-independent approaches have already provided important information about the extent of HGT in natural settings. Single-cell detection of donor, recipient and transconjugant bacteria in various natural environments, combined with individual-based mathematical models, has provided a new platform for HGT studies. Innovative studies might develop from this platform, offering a deeper glimpse into microbial evolution. Such an understanding will be instrumental in implementing timely precautions against future microbial epidemics and the development of rational strategies to prevent the evolution of multiresistant pathogenic bacteria.

STOMATA
Pores on the underside of leaves, which enable gas exchanges. They can be either opened or closed.

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Competing interests statement
The authors declare no competing financial interests.

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