Review article

Horizontal gene transfer as a biosafety issue: A natural phenomenon of public concern

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Abstract

The transfer of genetic information between distantly or even unrelated organisms during evolution had been inferred from nucleotide sequence comparisons. These studies provided circumstantial evidence that in rare cases genes had been laterally transmitted amongst organisms of the domains bacteria, archaea and eukarya. Laboratory-based studies confirmed that the gene pools of the various domains of organisms are linked. Amongst the bacterial gene exchange mechanisms transduction, transformation and conjugation, the latter was identified as the mechanism with potentially the broadest host range of transfer. Previously, the issue of horizontal gene transfer has become important in the context of biosafety. Gene transfer studies carried out under more natural conditions such as in model ecosystems or in the environment established that all gene transfer mechanisms worked under these conditions. Moreover, environmental hot-spots were identified where favourable conditions such as nutrient enrichment increased the probability of genetic exchange among bacteria. In particular, the phytosphere was shown to provide conducive conditions for conjugative gene exchange. Concern has been expressed that transfer of recombinant DNA (e.g. antibiotic resistance genes) from genetically modified organisms (GMOs) such as transgenic plants to phytosphere bacteria may occur and thus contribute to the undesirable spread of antibiotic resistance determinants. Studies which were performed to address this issue clearly showed that such a transfer occurs, if at all, at extremely low frequency. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

The issue of horizontal gene transfer (HGT) between closely, distantly or even unrelated or-

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ganisms is one of the most intensively studied fields in the bio-sciences since 1940. This is not only of interest with respect to our understanding of the evolutionary implications of horizontal gene exchange, but also became very important as part of the risk assessment of accidentally or deliberately released genetically modified organisms (GMOs) into the environment.

The lateral transmission of genetic information among bacteria is accomplished by one of the three distinctive transfer mechanisms, transduction, transformation or conjugation. Each transfer mechanism is characterised by its specific host range. In bacterial transduction, genetic information is transferred from donor to recipient cells by bacteriophages (Calendar, 1988). Since phages often display a very narrow host range of infection, gene exchange among distantly related bacteria by transduction is not considered to contribute substantially to the dissemination of genetic information across species boundaries. Transformation is defined as the uptake, integration and stable inheritance of cell free DNA by bacterial cells. The natural competence to act as recipients has been identified in several genera including *Acinetobacter*, *Haemophilus*, *Pneumococcus*, *Streptococcus*, *Bacillus*, *Pseudomonas* and *Neisseria* (Lorenz and Wackernagel, 1994). Since some of these bacterial species are promiscuous in the uptake of cell free DNA, HGT by transformation between even distantly related bacteria seems feasible. Successful gene transfer by both, transformation and transduction usually requires the incorporation of DNA into the bacterial chromosome to ensure stable maintenance. Conjugation which requires cell to cell contact is a process whereby plasmids or transposons transfer from donor to recipient cells (Clewell, 1993). Since many of the conjugative elements exhibit a broad host range (bhr) of transfer and autonomous replication, conjugation is regarded to be an important factor for gene flux among bacteria.

Conceptually, gene transfer events in nature have been assessed using three distinct approaches. The first one which is often the only way to detect gene transfer events across species boundaries, involves the detection of homologous (orthologous) genes in different organisms by nu-

cleotide or deduced amino-acid sequence analysis which do not follow the general pattern of evolutionary divergence of these organisms. The second approach is to demonstrate gene transfer experimentally under laboratory conditions, whereas the third one deals with the analysis of lateral transmission of genetic information under more natural conditions employing microcosms and field studies.

The present survey is subdivided in five parts and presents selected data about both circumstantial as well as direct evidence for HGT events. The first part deals with reports on gene transfer events between members of distantly related genera or even unrelated organisms as predicted by comparative DNA or deduced amino-acid sequence analysis. This includes interdomain transfer, e.g. between bacteria and plants or transkingdom transfer, e.g. between animals and plants, as well as intergeneric and interspecific transfer. The second part describes laboratory-based studies demonstrating links between the different gene pools of organisms. The third part presents in situ studies which analyse gene exchange among bacteria in an environmental hotspot of gene transfer, the phytosphere. The fourth part focuses on HGT from transgenic plants to members of the soil microbiota. Finally, the implications of a potential transfer of antibiotic resistance markers from transgenic plants to indigenous microorganisms are considered.

2. Circumstantial evidence indicates travelling of genes across species boundaries during evolution

The rapid progress in the recording of genetic information from various organisms led to a considerable accumulation of nucleotide sequence data. Thus detailed analyses on the distribution of homologous genes in different organisms became feasible. Accordingly, HGT events were analysed on basis of nucleotide or deduced amino-acid sequence homologies of selected genes. The corresponding results indicated lateral transmission of genes in rare cases during evolution.

In such a way HGT was debated about animals belonging to different fly species (Houck et al.,

Table 1
Horizontal gene transfer events which were proposed on basis of nucleic-acid or deduced amino-acid sequence homologies

Genetic element or gene	HGT event (transfer between)	Proposed organisms	References
P elements (mobile elements)	Interspecific (animals)	*D. melanogaster* and *D. willistoni*	Houck et al., 1991
papD (PapD chaperone)	Interdomain (human cells and bacteria)	Human cells and *E. coli*	Holmgren and Bränden, 1989
rol C[a]	Interdomain (plants and bacteria)	*Nicotiana* ancestor and *A. rhizogenes*	Furner et al., 1986; Meyer et al., 1995
cat (group II catalase)	Interdomain (fungi and bacteria)	Unknown	Klotz et al., 1997
pgi (glucose-6-phosphate isomerase)	Interdomain (eukaryotes and bacteria)	Unknown	Katz, 1996

[a] Located on the T_L-DNA of the Ri-plasmid of *A. rhizogenes*; *rol* genes are essential for tumorgenesis on various dicotyledonous plants.

1991), between animals and plants (Calvi et al., 1991), between different yeast genera (Hardy and Clark-Walker, 1991), between human cells and bacteria (Holmgren and Bränden, 1989), between plants and bacteria (Furner et al., 1986; Meyer et al., 1995), between fungi and bacteria (Klotz et al., 1997), between archaea and bacteria (Smith, 1994), between protists and bacteria (Smith et al., 1992) as well as between eukaryotes and bacteria (Katz, 1996). In the following part of this section some selected reports cited above are discussed in more detail. These examples are also summarised in Table 1.

A potential HGT event between animals belonging to different fly species was described by Houck et al. (1991). The authors reported on the transfer of a mobile P element from a species of the *Drosophila willistoni* group into *Drosophila melanogaster*. The hypothesis was based on the observation that: (i) only few *D. melanogaster* strains carry P elements (P strains) whereas others do not contain P elements (M strains); (ii) the abundance of P elements in species of the *willistoni* group relative to their paucity in the *melanogaster* species group; (iii) the near identity of P element sequences from *D. melanogaster* and *D. willistoni* which is not in accordance with the evolutionary distance of both groups; and (iv) the overlap in geographical ranges of *D. melanogaster* and *D. willistoni*. As a transfer vector the semiparasitic mite *Proctolaelaps regalis* was proposed. The geographic distribution of the mite corre-

sponds with that of *Drosophila*. Hence the mite is present in Florida, Central- and South-America, where the spread of both fly species overlaps. P-element sequences were identified in P strain associated *P. regalis* mites but not in the *P. regalis* mites which are associated with M strains. In addition, mites which are closely related to *P. regalis* lacked P element sequences. Fly specific ribosomal DNA sequences were identified in both, P and M strain associated mites but were absent in closely related mites. It was hypothesised that *P. regalis* acquired P elements during feeding and was therefore able to transfer the mobile elements between *D. melanogaster* and *D. willistoni* species. Since the mite feeds on all immature stages on both, *D. melanogaster* and *D. willistoni* species, transfer of P elements may have occurred by taking up DNA from an egg of *D. willistoni* and injecting it into an egg of *D. melanogaster*. In summary, the authors concluded that HGT from *Drosophila* to the mite and in the opposite direction may have occurred.

The amino-acid sequence homology between the *Escherichia coli* PapD protein, which is involved in the process of attachment of pathogenic *E. coli* isolates to human cells of the urinary tract and the human lymphocyte differentiation antigen Leu1/CD5 indicates a HGT event between bacteria and human cells (Holmgren and Bränden, 1989). Amino-acid residues -4–252 of the Leu-1 protein displayed a 26% identity when compared with the sequence of the complete PapD molecule.

The CD5 proteins evolve quite fast, for example CD5 proteins of the closely related human and mouse show sequence identity ranging from 43% in domain 1 to 58% in domain 3 of the protein. Hence, the possibility of divergent evolution of PapD and Leu-1/CD5 from a common ancestor was considered very unlikely. More likely, inter-domain gene transfer had occurred at a much later stage in evolution. These findings suggest that genes involved in eukaryotic cell–cell interactions were recruited by bacteria to assist in their attachment to eukaryotic cells.

An evidence that naturally occurring gene transfer mediated by *Agrobacterium* may lead to naturally made transgenic plants was brought by Furner et al. (1986). The authors reported that the genome of the tobacco plant *Nicotiana glauca* contains *rol* genes which are also present on the single copy T_L-DNA of *Agrobacterium rhizogenes*. The comparison of the deduced polypeptides encoded by both, the prokaryotic and the eukaryotic *rolC* gene, revealed a homology of 75%. Southern hybridisation analyses performed by these authors and by Meyer et al. (1995) revealed that *rol* genes are only present in species of the genus *Nicotiana* which derived from a common ancestral complex (*Cestoid*). Hence, an interdomain transfer from *A. rhizogenes* to the ancestor of the plants containing *rol* genes which belonged to the *Cestoid* complex may have occurred. The direction of this HGT event seems plausible since the *N. glauca rol* genes are present as an imperfect inverted repeat which is frequently located in T-DNA transformed cells. Transformed plant cells containing *rol* genes can be readily generated to whole plants in vitro, leading to the hypothesis that such an event may have occurred in nature.

Klotz et al. (1997) compared the sequences of 70 catalase proteins of bacterial, fungal, animal as well as of plant origin. The resulting phylogenetic tree revealed six distinct groups comprising plant catalases, animal catalases, two groups containing only bacterial catalases, one group of fungal catalases and, interestingly, one group containing fungal as well as bacterial catalases. The latter group included organisms such as the ascomycetes *Aspergillus niger* and *A. nidulans*, the low GC Gram-positive bacteria *Bacillus subtilis* and *B. firmus* or

Pseudomonas putida as a member of the γ-proteobacteria. Hence, the authors speculated about HGT events between bacteria and fungi. It is worth noting that some of the organisms of the corresponding group are known to live in close interaction with each other on plant hosts.

There are two classes of bacterial phosphoglucose isomerase (PGI) genes. One class is represented by the *E. coli* sequence (Froman et al., 1989), the other by the sequence of *Zymomonas mobilis* (Hesman et al., 1991). Both sequences differ from each other at 52% of their inferred amino acid sequences. Furthermore, the *E. coli* and the *Z. mobilis* amino acid sequences differ from the corresponding mammalian sequence at 30% and 50%, respectively. Recently, Katz (1996) presented phylogenetic analysis of 28 different PGI sequences of species belonging to protists, plants, fungi, animals as well as bacteria. This study confirmed that some bacterial PGI sequences including that of *E. coli* are more closely related to eukaryotic organisms than to other bacterial species. Furthermore, molecular clock estimates indicated a divergence time of 470–650 million years between the *E. coli* and animal sequences, which is much shorter than the proposed divergence time between eukaryotes and bacteria. In summary, the authors speculated that a *pgi* gene had been laterally transmitted between an eukaryote and a bacterium.

3. Laboratory-based studies demonstrate gene transfer among organisms of the domains bacteria, archaea and eukarya

The reports cited in the first part of this survey suggest that HGT even between organisms belonging to different organism domains took place during evolution. Moreover, they suggest linkage of the gene pools of organisms of the three domains bacteria, archaea and eukarya. In order to obtain experimental proof upon the existence of such a linkage many laboratory based studies were conducted. These studies focused mainly on gene transfer by conjugation, due to the extraordinary promiscuity of conjugative processes. The examples cited below are summarised in Table 2.

Table 2
Selected studies providing experimental evidence of gene exchange among organisms of the domains bacteria, archaea and eukarya

Genetic element(s)	HGT event (mechanism)	Transfer between	References
Promiscuous conjugative plasmids of, e.g. incompatibility groups IncW, P, N	Interspecific/intergeneric (conjugation)	Most Gram-negative bacterial species	Thomas, 1989
Conjugative broad host range plasmids pAMβ1, pIP501 and pIJ101	Interspecific/intergeneric (conjugation)	Gram-positive bacterial species, e.g. from Streptococcus to Bacillus, Clostridium, Lactobacillus, Listera, Staphylococcus (pAMβ1)	Clewell et al., 1974; Horodniceanu et al., 1976; Kieser et al., 1982
Gram-negative/Gram-positive shuttle plasmids which contained the origin of replication of both, the donor and recipient bacterial species; transfer was mediated by either a Gram-positive or a Gram-negative plasmid encoded transfer system	Intergeneric (conjugation)	Gram-positive and Gram-negative bacterial species, e.g. from E. coli to Streptomyces species, E. coli to coryneform bacteria, E. coli to E. faecalis; E. faecalis to E. coli	Trieu-Cuot et al., 1988; Mazodier et al., 1989; Schäfer et al., 1990; Williams et al., 1990
Mobilisable IncQ plasmid RSF1010; mobilisation was mediated by the transfer functions of IncPα plasmid RP4.	Intergeneric (conjugation)	Gram-positive and Gram-negative bacterial species: from E. coli to M. smegmatis or S. lividans	Gormley and Davies, 1991
Conjugative transposons (Tn916 and related)	Intergeneric (conjugation)	Gram-positive and Gram-negative bacterial species, e.g. from E. faecalis to C. freudii and from E. coli to B. subtilis	Bertram et al., 1991; Clewell and Flannagan, 1993
Conjugative plasmid pNOB8	Interspecific (conjugation)	Members of the domain archea (between different Sulfolobus species)	Schleper et al., 1995
Ti-plasmid of A. tumefaciens	Interdomain (conjugation related process)	A. tumefaciens and many dicotyledonous as well as some monocotyledonous plants	Zambryski, 1992
Plasmids containing the mobilisation functions of the naturally occurring plasmid RSF1010; transfer was mediated by Agrobacterium vir functions	Interdomain (conjugation related process)	A. tumefaciens and plant cells	Buchanan-Wollaston et al., 1987
E. coli/yeast shuttle plasmid containing the origin of transfer of ColE1; transfer was mediated by the transfer functions of an IncPβ plasmid carrying the ColE1 mobC region	Interdomain (conjugation related process)	E. coli and S. cerevisiae	Heinemann and Sprague Jr., 1989
Ascobolus mitochondrial plasmid pAI2	Intergeneric (remains to be determined)	The fungi A. immersus and P. anserina	Kempken, 1995
E. coli cloning vector carrying the entire copy of poliovirus1	Interdomain (remains to be determined)	E. coli and animal cells ('Buffalo Green Monkey BGM')	Heitmann and Lopes-Pila, 1993

Conjugative plasmids have been identified in a wide range of Gram-negative and Gram-positive bacterial species. Plasmids of Gram-negative bacteria have been classified into more than 25 different incompatibility groups (Couturier et al., 1988), which differ in their host range of transfer and autonomous replication, pilus structure and size. There are some promiscuous plasmid species, for example those which belong to the incompatibility groups IncN, IncP and IncW. These plasmids usually transfer between most Gram-negative bacterial species (Thomas, 1989). Due to their possible potential to mobilise chromosomal sequences, conjugative plasmids, especially bhr vectors (e.g. R68.45, Haas and Holloway, 1976), are potential vehicles for gene flux within the Gram-negative bacterial groups.

Conjugative plasmids have also been described in a wide range of Gram-positive genera, including *Bacillus*, *Clostridium*, *Lactobacillus*, *Listera*, *Staphylococcus*, *Streptococcus–Enterococcus–Lactococcus* and *Streptomyces*. Some of these exhibit a bhr, for example pAMβ1 (Clewell et al., 1974) and pIP501 (Horodniceanu et al., 1976) which were originally found in the enterococcus group, or pIJ101 (Kieser et al., 1982), a small multicopy bhr vector originally identified in *Streptomyces lividans*. The existence of these bhr systems indicates that there is gene flux within the Gram-positive bacterial groups.

Conjugation between Gram-positive and Gram-negative bacteria has been demonstrated in laboratory-based studies. In most cases in vitro constructed shuttle vectors were used which contained the origin of replication for both, the donor and recipient bacterial species. Transfer was mediated by the aid of either a Gram-positive or a Gram-negative plasmid encoded transfer system. Thus, intergeneric conjugation was demonstrated from *E. coli* to *Streptomyces* species (Mazodier et al., 1989), to coryneform bacteria (Schäfer et al., 1990) as well as to a wide range of Gram-positive organisms (Trieu-Cuot et al., 1987; Williams et al., 1990). Conversely, transfer from *Enterococcus faecalis* to *E. coli* was also observed (Trieu-Cuot et al., 1988). An example involving conjugal transfer of a natural occurring plasmid between Gram-positive and Gram-negative bacteria is the transfer of the mobilisable IncQ plasmid RSF1010 from *E. coli* to *Mycobacterium smegmatis* and *S. lividans* (Gormley and Davies, 1991). The plasmid was stable with respect to structure and inheritance and conferred high-level resistance to streptomycin and sulfonamide in the Gram-positive host species.

Other important mobile elements which are of interest with respect to gene exchange between Gram-negative and Gram-positive bacteria are Tn916 and related conjugative transposons (Clewell and Flannagan, 1993). These elements display also bhr and have been introduced into various bacterial species via conjugation. For example, transfer of conjugative transposon Tn916 located on a pAD1 derivative was reported to occur from *E. faecalis* to the Gram-negative bacteria *Alcaligenes eutrophus*, *Citrobacter freudii* and *E. coli* and even back from an *E. coli* donor harbouring Tn916 on plasmid pBR322 to *B. subtilis*, *Clostridium acetobutylicum*, *E. faecalis* and *Streptococcus lactis* subsp. *diacetylactis* (Bertram et al., 1991).

Amongst members of the domain archaea one case of plasmid-mediated conjugation was reported. Schleper et al. (1995) identified the 45 kb multicopy plasmid pNOB8 which is present in the heterotrophic *Sulfolobus* isolate NOB8H2 at a copy number of 20–40 plasmid copies per chromosome. This plasmid transferred in a cell contact dependent manner from the isolate to strains of other *Sulfolobus* species such as *S. solfataricus* or *S. islandicus*. Plasmid pNOB8 confers no selectable phenotypes to its host but strains harbouring the plasmid display a lower growth rate and form small colonies when plated. Transfer appeared to occur rapidly and/or at a high frequency, because no recipient cells devoid of the plasmid were recovered from mating mixtures 2 days after beginning of mating.

The importance of conjugative processes for gene flux between organisms belonging to even different domains is evident from the naturally occurring transfer of genetic information from *Agrobacterium tumefaciens* to many dicotyledonous and some monocotyledonous plants (Zambryski, 1992). Thereby, a specific DNA fragment designated T-DNA (transferred DNA) lo-

cated on a 200-kbp plasmid (Ti-Plasmid), is transferred into the plant cell. The 25 kbp T-DNA is bordered by 23 bp direct repeat sequences termed left and right borders. These borders are the targets where specific cleavage occurs to generate T-intermediates in the *Agrobacterium* cell. These intermediates are transferred by yet unknown processes into the plant cell and incorporated into the plant genome. A prerequisite for the transmission of the T-intermediates is the close interaction of the bacterial cells with the plant cells. Apart from this contact dependence it is interesting that the *Agrobacterium* mediated gene transfer resembles a conjugative process, since many of the proteins essential for the transfer of the T-DNA are highly homologous to the transfer proteins encoded by conjugative plasmids belonging to the incompatibility group IncP (Farrand, 1993). Buchanan-Wollaston et al. (1987) reported that plasmids containing the mobilisation functions of IncQ plasmid RSF1010, a plasmid that is readily mobilised by IncP plasmids, can be transferred from *A. tumefaciens* into plant cells in the presence of the *A. tumefaciens* virulence (*vir*) functions. These are located on the Ti-plasmid and mediate the transfer of T-DNA. This result indicates that plants have an access to the gene pool of Gram-negative bacteria and underlines the importance of conjugative processes with respect to gene exchange crossing domain boundaries.

Another example for interdomain gene transfer by conjugation was first reported by Heinemann and Sprague Jr. (1989). The authors reported on the transfer of an *E. coli*/yeast shuttle plasmid, from *E. coli* to *Saccharomyces cerevisiae*. The shuttle plasmid contained the origin of transfer of ColE1. Transfer was mediated by the transfer functions of an IncPβ plasmid containing the ColE1 *mobC* region. Likewise, a shuttle plasmid transferred between *E. coli* and *S. cerevisiae*, mediated by the F-plasmid transfer machinery. Nishikawa et al. (1992) reported that such a gene transfer event is possible by using the IncQ (RSF1010) *mob* region within shuttle vectors and the helper plasmid pRK2013, too. Conjugative plasmid transfer occurred from *E. coli* into the yeast species *S. cerevisiae*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Pichia angusta*

(*Hansenula polymorpha*), and *Pachysolen tannophilus* (Hayman and Bolen, 1993).

In addition to studies which dealt with gene transfer mediated by conjugation several other studies were conducted to analyse HGT between even distantly related organisms under laboratory conditions. Thereby, the transfer observed differed from conjugative processes.

Among fungi, transfer of circular and linear plasmids has been observed in the genus *Neurospora* (Collins and Saville, 1990; Griffiths et al., 1990; Debets et al., 1994). HGT between unrelated fungi was reported by Kempken (1995). The fungus *Ascobolus immersus* which is known to contain several linear plasmids was used as the donor and *Podospora anserina* as a plasmidless recipient. Upon hyphal contact, transfer of the *Ascobolus* mitochondrial plasmid pAI2 into *Podospora* was detected.

In order to investigate interdomain gene transfer between bacterial and animal cells, Heitmann and Lopes-Pila (1993) analysed the transfer of an *E. coli* cloning vector carrying an entire copy of poliovirus1 from *E. coli* to 'Buffalo Green Monkey BGM' cells. The successful transfer was detected by the expression of the genetic information of the virus in the recipient cell leading to cell lysis. The frequency of gene transfer events was in the range of 10^{-5} per bacterial donor cell. Further analyses revealed the resistance of the mechanism of DNA uptake against DNaseI and the necessity of living bacterial cells for successful HGT. The authors speculated about phagocytosis as a potential transfer mechanism. Another example of gene transfer between bacteria and mammalian cells was reported by Courvalin et al. (1995). Replicative as well as integrative vectors were efficiently transferred from invasive *Shigella flexneri* and *E. coli* strains to mammalian cells. This direct transfer displayed a broad host cell range and the vectors were stably inherited and expressed by the cell progeny.

4. The phytosphere is conducive for the horizontal transmission of genetic information among bacteria

With the pending release of GMOs into the

environment gene transfer analyses have become an important issue for biosafety reasons. Concern has been expressed that recombinant DNA introduced into GMOs may be transferred to autochthonous microorganisms. Consequently, numerous studies have been initiated with the objective to analyse gene transfer in natural environments. Several studies focusing on bacteria established the existence of environmental hot-spots which are conducive for HGT events. In particular the phytosphere i.e. the phylloplane, the phyllosphere, the rhizoplane, as well as the rhizosphere have been shown to provide favourable conditions for gene exchange among bacteria (Table 3).

Transfer of bhr IncPα plasmid RP4 between *Pseudomonas* donor and recipient strains was studied in wheat rhizosphere and non-rhizosphere soil by van Elsas et al. (1989). Transfer of RP4 was demonstrated, but only in nutrient amended non-rhizosphere soil or in rhizosphere soil. Additionally, van Elsas et al. (1988) reported that both, the survival of *Pseudomonas* sp. RF2 donor (RP4) and recipient cells as well as the plasmid transfer frequencies decreased drastically at increasing distances from the roots.

Plasmid transfer under field conditions between donor and recipient strains introduced in the sugar beet rhizosphere was demonstrated by Lilley et al. (1994). *Pseudomonas marginalis* 376N harbouring the conjugative mercury resistance plasmid pQBR11 was used as donor and *Pseudomonas aureofaciens* 381R as recipient strain. Plasmid transfer could be detected at frequencies ranging from 5.1×10^{-5} to 1.3×10^{-8} transconjugants per recipient cell after 24 h of incubation in the rhizosphere and from 1.3×10^{-2} to 1.7×10^{-6} on the peel of the sugar beet roots. No transfer of the mercury resistance plasmid was detected between bacteria which were placed in soil at distances of more than 5 cm from plants.

Troxler et al. (1997) showed that bhr plasmid mediated mobilisation of chromosomal genes between introduced pseudomonads occurred in soil microcosms. The auxotrophic *Pseudomonas aeruginosa* strain PAO harbouring the conjugative plasmid R68.45 displaying chromosome mobilising ability (cma) as the donor and a *P. aeruginosa* PAO derivative carrying different auxotrophic markers as recipient, were co-inoculated in microcosms containing nonsterile soil and wheat plants. After 2 weeks of incubation chromosomal recombinants of the recipient were detected at a frequency of 8.0×10^{-7} recombinants per donor. In contrast, no transfer of chromosomal genes could be observed within 14 days of inoculation in nonsterile soil without plants.

These selected studies clearly showed that the rhizosphere of plants is conducive for conjugative gene transfer compared with bulk soil and may be regarded as a hot-spot for conjugation in the terrestrial environment. It is now well established that nutrient enrichment of soil either through sterilisation, which increases levels of available organic carbon and nitrogen, by direct amendment or by the presence of a plant rhizosphere facilitates conjugative transfer in soil. Nutrient amended soil allows introduced inoculants to colonise and grow in the system without competition and interactions with the natural microbial population (Wellington et al., 1993).

Conjugative plasmid transfer between two *Sinorhizobium meliloti* (former name *Rhizobium meliloti*, de Lajudie et al., 1994) 2011 derivatives in nodules of alfalfa was investigated by Pretorius-Güth et al. (1990). The induction of nitrogen fixing root nodules on alfalfa by *S. meliloti* requires the genetic functions encoded by two different megaplasmids designated pM1 and pM2. The authors constructed a *S. meliloti* donor strain harbouring an intact pM1, a mobilisable pM2 derivative containing the RP4 *mob* region on a modified transposon Tn5 inserted into the *Inf* region as well as an IncPα helperplasmid (RP4-4). Hence, this donor strain was infection deficient (Inf⁻) and induced ineffective nodules on alfalfa roots. The recipient strain harboured an intact pM2 and a modified pM1 containing a transposon Tn5 derivative inserted in the *Nod* region. Nod⁻ strains are unable to induce root nodules on alfalfa plants. Co-inoculation of alfalfa seedlings with both, the Inf⁻ strain as well as the Nod⁻ strain resulted in the formation of wildtype nodules on alfalfa roots. Thus, the strategy em-

Table 3
Selected studies which demonstrate that the phytosphere is an environmental hot-spot of HGT

Plant environment	Transfer mechanism	Relevant observation	References
Rhizosphere (sugar beet or wheat)	Conjugation	Conjugative gene transfer between introduced donor and recipient cells occurred readily in the plant rhizosphere but was drastically reduced or below the limit of detection in the corresponding non-rhizosphere soil.	van Elsas et al., 1988, 1989; Lilley et al., 1994; Troxler et al., 1997
Root nodules (alfalfa)	Conjugation	Megaplasmid pM2 of *S. meliloti* transferred between donor and recipient cells inhabiting the same alfalfa root nodule as frequently as in filter matings (approx. 10^{-5} per donor cell).	Pretorius-Güth et al., 1990
Phytosphere (sugar beet)	Conjugation	Transfer of mercury resistance plasmids from the indigenous microbiota to a released *Pseudomonas* strain colonising the plant phytosphere occurred 88 days after sowing at high frequencies ranging from 1.3×10^{-2} to 1 per recipient cell.	Lilley and Bailey, 1997
Phyllosphere (bush bean)	Conjugation	The phyllosphere supports conjugation by the nutrients supplied by the leaf. High frequency conjugation of IncPα plasmid RPI between added donor and recipient cells was observed. One day after inoculation, numbers of transconjugants reached 10% of the donor viable count.	Björklöf et al., 1995
Phylloplane (soybean or bean)	Transduction	After co-inoculation of a nalidixic-acid resistant (Nal^r) *P. aeruginosa* strain lysogenic for the generalised transducing bacteriophage F116 and a *P. aeruginosa* strain containing a low-copy carbenicillin resistance (Cb^r) plasmid on sterile bean and soybean leaves, transductants (Nal^r, Cb^r) were observed on 90% of the leaves studied.	Kidambi et al., 1994
In planta (maize)	Conjugation	Transfer of plasmid RP1 between *E. crysanthemi* strains occurred at high transfer frequencies in the range of 10^{-2}–10^{-3} after separately injection of approximately 10^{7} donor and recipient cells into maize. These frequencies were two to three orders of magnitude higher than those obtained in parallel in vitro matings.	Lacy, 1978

ployed ensured that wildtype nodules which were induced on alfalfa plants contained both, the donor and the recipient strain. Hence, mobilisation of the modified pM2 from the donor to the recipient strain harbouring the defective pM1 could be investigated in effective alfalfa nodules (in situ). The results demonstrated that HGT via conjugation occurred within the nodules. Moreover, transfer of pM2 occurred as frequently in the nodules (in situ) as in filter mating experiments (in vitro). This indicates that the root nodule represents a favourable environment for conjugative gene exchange between *S. meliloti*.

Transfer of mercury resistance plasmids from the indigenous microflora to a genetically tagged *Pseudomonas fluorescence* strain (*lacZY*; *aph*, encoding aminoglycoside 3′-phosphotransferase) has been observed in field experiments by Lilley and Bailey (1997). The strain was introduced as a seed dressing to sugar beets and monitored for the acquisition of mercury resistance plasmids. Interestingly, transconjugants harbouring genetically distinctive mercury resistance plasmids were detected at high frequencies in the range of $1.3 \times 10^{-2}–1$ in root and leaf samples. Transconjugants were shown to occur at a specific period of plant development, following the emergence of the first foliage leaves.

Björklöf et al. (1995) reported that conjugative transfer of the IncPα plasmid RP1 between epiphytically growing *Pseudomonas syringae* donor and recipient strains occurred in the phyllosphere of bush bean (*Phaseolus vulgaris*) at high frequencies (10^{-1} per donor cell) under high humidity conditions. The observed high frequency conjugation was supported by both, the available nutrients provided by the leaf and also, to a lesser extent, by the leaf surface itself. Thus, the microhabitat phyllosphere is conducive for HGT events via conjugation.

Evidence for phage mediated gene transfer (transduction) among *P. aeruginosa* strains on the phylloplane was reported by Kidambi et al. (1994). The authors co-inoculated a nalidixic-acid resistant (Nal[r]) *P. aeruginosa* strain lysogen of the generalised transducing bacteriophage F116 and a *P. aeruginosa* strain containing a low-copy carbenicillin resistance (Cb[r]) plasmid on sterile bean and

soybean leaves. In this experiment, the F116 lysogenic strain was used as the source of virus particles and also served as the recipient of transduced plasmid DNA. Following inoculation, transductants (Nal[r], Cb[r]) were observed on 90% of the leaves studied.

In planta transfer of plasmid RP1 between *Erwinia crysanthemi* strains which were inoculated into maize was described by Lacy (1978). High transfer frequencies in the range of $10^{-2}–10^{-3}$ were observed after separate injection of approximately 10^7 donor and 10^7 recipient cells, respectively. Interestingly, these frequencies were two to three orders of magnitude higher than those obtained in parallel in vitro matings. Additionally, Lacy et al. (1984) demonstrated intergeneric transfer of plasmid RP1 between *Erwinia herbicola* or *P. syringae* donors and an *Erwinia amylovora* recipient on detached pear blossoms. In some cases the observed transfer frequency was as high as 10^{-1}.

In conclusion, the results of the above described studies suggest that the phytosphere provides favourable conditions for the exchange of genetic material among bacteria. This is probably due to the fact that the plant contains microhabitats where the nutrient status is favourable for the exchange of genetic information. The rhizosphere and the rhizoplane provide nutrients by root exudation of organic molecules through the root cell wall and by the sloughing off of root cells while the growing of the root into the soil (Wellington et al., 1993). Another energy source is the simple death of roots and following lysis of plant cells. The phyllosphere and phylloplane is conducive to the exchange of genetic material under natural conditions which frequently occur in nature. Björklöf et al. (1995) reported that high humidity causes a leaching of nutrients from the leaves which are then accessible for the microbes on the surface. Furthermore, they found that the leaf surface itself stimulates conjugation.

5. Transfer of recombinant genes from transgenic plants to plant associated or indigenous soil bacteria occurs, if at all, at extremely low frequencies

Since the exchange of genetic material between

even distantly related organisms is possible, the issue arose to what extent transfer of recombinant DNA from transgenic plants to indigenous soil microorganisms may occur. Particular concern has been expressed that transgenic plants carrying antibiotic resistance genes may transfer those genes to the soil bacterial community. Selectable antibiotic resistance markers which are used for the construction of transgenic plants include genes encoding resistance to kanamycin/neomycin/geneticin, hygromycin B, methotrexate, gentamicin, bleomycin or phosphinotricin. The most commonly used marker gene is the Tn5 derived *nptII* gene (Beck et al., 1982) which confers resistance to kanamycin, neomycin and geneticin (G418). More than 100 laboratories around the world have used the *nptII* gene in introducing recombinant DNA into more than 30 different plant species such as tomato, potato, pea, kiwi or cotton (Flavell et al., 1992).

One prerequisite for HGT from a plant to an indigenous soil microorganism is its opportunity to come in close contact with plant DNA. This is either facilitated by ploughing the plants into the soil and following lysis of the plant cell or by bacterial colonisation of whole plants. Of particular interest are plant pathogenic bacteria which are able to destroy or alter the plant tissue. DNA which is released into the soil is a potential target of soil-borne nucleases. However, it is also protected by its rapid and extensive binding on solid material such as clay minerals, quartz, feldspar, heavy minerals as well as humic acids (Lorenz and Wackernagel, 1994). A second prerequisite is the ability of the microorganism to take up DNA (natural transformation). The natural competence of soil bacteria to act as recipients has been identified in several genera including *Acinetobacter*, *Azotobacter*, *Bacillus*, *Pseudomonas*, *Rhizobium* or *Streptomyces* (Lorenz and Wackernagel, 1994). Moreover, it has been shown that gene transfer via transformation occurs under in situ conditions. For detailed information the reader is referred to the reviews of Stewart and Carlson (1986), Stewart (1992), as well as Lorenz and Wackernagel (1994). However, if soil microorganisms come in close contact with recombinant DNA, gene transfer may occur. Consequently sev-

eral studies addressed this issue mainly focusing on the transfer of antibiotic resistance determinants from the plant to the soil microbiota under in situ conditions. Most studies mentioned below are listed in Table 4.

Prior to the commercial sales of its genetically engineered FLAVR SAVR tomato, Calgene, CA, conducted a thorough review on the biosafety of the transgenic tomato (Calgene, 1990; Redenbaugh et al., 1994). As part of these analyses, questions on the use of the *nptII* gene and its gene product, the aminoglycoside 3′-phosphotransferase II (APH(3′)II) were addressed. One important issue was the probability of a HGT of the marker gene from the plant to indigenous soil bacteria. A theoretical gene transfer model was developed which based on the putative transfer from the plant to the Gram-positive *B. subtillis* which has a natural transformation system, but displays no homology to the recombinant DNA. Alternatively, transfer of recombinant DNA from the plant to *A. tumefaciens* was considered which has no known natural transformation system but harbours the Ti-plasmid carrying the same T-DNA border regions inserted into the plant genome. The models estimated a 'worst case' scenario (e.g. 100% of soil bacteria are transformable, every fragment of the plant DNA in soil contains the *nptII* sequence, the transformed gene is expressed after integration into the recipient genome and the gene product is active and stable in the bacterial cells) or the 'more likely' scenario (e.g. only 10% of soil bacteria are transformable, the *nptII* gene constitutes the 10^{-5} part of the size of the tomato genome, the gene is not always integrated and expressed). For the *Bacillus* type transformation system 9×10^5 transformants per acre in the worst case scenario were estimated and two transformants per acre in the more likely case. For the *Agrobacterium* type transformation system the estimates were three orders of magnitude less. From these calculations it can be concluded that in the worst case kanamycin resistant *Bacillus* transformants will constitute approx. the 10^{-7} part of kanamycin resistant soil bacteria. In the case of *Agrobacterium* predicted transformants will constitute the 10^{-10} part of the kanamycin

Table 4
Selected studies which analysed HGT from plants to bacteria under laboratory conditions or in field experiments

HGT between	Experimental procedure	Transfer detected?	References
Transgenic tobacco and the plant pathogen *A. tumefaciens*	*A. tumefaciens* was incubated with leave disks of transgenic tobacco plants containing the *aacC1* gene conferring gentamicin resistance. After reisolation from the plant tumour, *Agrobacterium* was tested for the presence[a] of the transgene.	No	Broer et al., 1996
Transgenic potato and the plant pathogen *E. crysanthemi*	Transgenic potato plants containing the Tn*3* encoded β-lactamase gene (*bla*) were co-cultivated with *E. crysanthemi*. After reisolation from potato tissue, selection was made for ampicillin-resistant *E. crysanthemi*.	No	Schlüter et al., 1995
Transgenic sugar beet and indigenous soil bacteria	After transgenic sugar beets containing the *nptII* gene had been ploughed into the soil in a field experiment, indigenous soil bacteria were tested for the presence[b] of the *nptII* gene.	No	Smalla et al., 1994
Transgenic tobacco and indigenous soil bacteria	One year after the harvest of transgenic tobacco containing the *aacC1* gene encoding gentamicin resistance, indigenous soil bacteria were tested for the presence[c] of the transgene.	No	Paget and Simonet, 1994
Transgenic *Brassicaceae* and the fungus *A. niger*	Transgenic plants harbouring, e.g. pUC18 plasmid derivatives containing a hygromycin B resistance gene were co-cultivated with the fungus in soil. In one case a fungal transformant which contained pUC18 sequences was obtained[d].	Yes	Hoffmann et al., 1994

[a] *Agrobacterium* cells isolated from the tumour tissue were plated on agar plates containing gentamicin. Gentamicin-resistant clones were analysed in Southern hybridisation experiments using the *aacC1* gene as a probe.

[b] Soil extracts were plated on plate count agar supplemented with varying concentrations of kanamycin. Kanamycin-resistant colonies were tested for the presence of the *nptII* gene in dot-blot hybridisation experiments. Positive clones were analysed by PCR using transgene specific primers.

[c] Indigenous soil bacteria were isolated from soil, total DNA was extracted and PCR amplification was performed using transgene specific primers.

[d] Fungal progeny was isolated and plated on agar plates containing hygromycin B for selection of putative transformants. After 3 weeks, conidiospores of growing colonies were subcultured on fresh agar plates containing hygromycin B. Southern hybridisation experiments revealed the occurrence of the hygromycin B resistant determinant in ten out of approximately 200 hygromycin B resistant colonies which were obtained after co-cultivation with different transgenic plants (*B. napus*, *B. nigra*, *D. innoxia*, as well as *V. narbonensis*). Only in one case the transgenic character of a resistant colony could be stably maintained after continuous subculture of conidiospores. This colony was obtained after co-cultivation of a transgenic *D. innoxia* plant harbouring a pUC18 derivative carrying a CaMV35S controlled hygromycin B resistance gene. Parts of the hygromycin B resistance plasmid originating from the plant (a plasmid derivative without the hygromycin B resistant determinant) could be re-cloned from transformant total DNA.

resistant soil bacteria if the worst case scenario is regarded.

Broer et al. (1996) analysed HGT from transgenic tobacco to the plant pathogen *A. tumefaciens*. The pathogen was incubated with leave disks of transgenic tobacco plants containing the *aacC1* gene conferring gentamicin resistance to bacteria. Furthermore, the plants harboured the

luciferase (*luc*) gene driven by the constitutive Cauliflower Mosaic Virus promotor CaMV35S, which mediates bioluminescence expression in plants and bacteria. After plant tumours arose, *Agrobacterium* cells were isolated from the tumour tissue and plated on agar plates containing gentamicin. Of 4000 gentamicin-resistant clones which were obtained none exhibited a bioluminescent phenotype. Southern hybridisation analysis of selected gentamicin-resistant clones revealed that these clones were spontaneously resistant to gentamicin, since no *aacC1* gene could be detected. Hence, no HGT event from transgenic tobacco to *Agrobacterium* had occurred.

Gene transfer from transgenic potato plants to the plant pathogen *E. chrysanthemi* was studied by Schlüter et al. (1995). Transgenic potato plants containing a pBR322 fragment carrying the Tn*3* encoded β-lactamase gene (*bla*) and the pBR322 origin of replication (*oriV*) were co-cultivated with *E. chrysanthemi*. After reisolation from potato tissue, selection was made for ampicillin resistance. Despite of the close interaction between the plant tissue and the pathogen, no ampicillin-resistant *E. crysanthemi* were obtained.

Smalla et al. (1994) investigated HGT from transgenic sugar beets to the indigenous soil bacterial community during a field experiment. After the transgenic plants containing a *nptII* gene conferring kanamycin-resistance in bacteria had been ploughed into the soil, indigenous bacteria were tested for the presence of this gene. Soil extracts were plated on plate count agar supplemented with varying concentrations of kanamycin. Kanamycin-resistant colonies were tested for the presence of the transgene originating from the plant in dot-blot hybridisation experiments. Positive clones were analysed by PCR using primers specific for the *nptII* gene of the transgenic plants. No HGT from the plants to indigenous soil bacteria was detected. Half a year after the plants have been ploughed into soil, PCR experiments still revealed the presence of the transgenic DNA in soil.

HGT from transgenic tobacco to the indigenous soil bacterial community was analysed by Paget and Simonet (1994). One year after the harvest of transgenic tobacco containing the *aacC1* gene encoding gentamicin resistance, indigenous soil bacteria were tested for the presence of the transgene. Soil bacteria were isolated, total DNA was extracted and PCR experiments were performed using transgene specific primers. No transfer of the *aacC1* gene to the indigenous soil bacterial community could be detected.

Interdomain gene transfer from a transgenic plant to a microorganism was reported by Hoffmann et al. (1994). HGT of a hygromycin B resistance gene which was under the control of a constitutive Cauliflower Mosaic Virus promotor (CaMV35S) from different transgenic plants to various soil microorganisms was analysed in co-inoculation experiments. Employing the fungus *A. niger* as recipient, mycelium was inoculated into sterile moist soil. Sterile transgenic plants (*Brassica napus*, *Brassica nigra*, *Datura innoxia*, as well as *Vicia narbonensis*) were added and grown together with the fungus for several weeks. Fungal progeny was then isolated and plated onto agar plates containing hygromycin B for selection of putative transformants. After 3 weeks conidiospores of growing colonies were subcultured on fresh selective agar plates. Southern hybridisation experiments revealed the presence of the hygromycin B resistant determinant in ten of approximately 200 resistant colonies obtained after co-culture with the different transgenic plants. However, only in one case the transgenic character of a resistant colony could be stably maintained after continuous subculture of conidiospores. This colony was obtained after co-cultivation of a transgenic *D. innoxia* plant which harboured a pUC18 derivative carrying the CaMV35S controlled hygromycin B resistance gene. Parts of the resistance plasmid originating from the plant (a plasmid derivative without the hygromycin B resistant determinant) could be recovered in *E. coli* transformed with *Cla*I restricted and religated fungal transformant total DNA. The ability of *A. niger* to act as recipient in transformation experiments using plasmid DNA inoculated into the soil was confirmed. Thus, HGT from plants to *A. niger* may have occurred in the soil.

In conclusion, studies which analysed gene transfer from plants to indigenous soil microor-

ganisms under in situ conditions revealed that if such events occur at all, they occur with extremely low frequencies which are often below the limit of detection. However, it should be mentioned that in those cases where bacterial cells were co-inoculated with transgenic plants, recipient cells did not contain DNA sequences homologues to those bordering the transgene. The presence of homologues sequences would facilitate establishment of the transgene in the recipient, thus increasing the probability of the detection of HGT events.

6. Conclusions

As outlined in this survey circumstantial as well as experimental evidence indicates that HGT between closely, distantly or even unrelated organisms occurs in nature, i.e. that the gene pools of the different organism domains are connected. However, successful interdomain or transkindom HGT seems to be a rather rare event in evolutionary times. Theoretical models as well as experimental studies indicate that transfer of recombinant DNA from transgenic plants to plant associated microorganisms occurs, if at all, at very low frequency. Nevertheless, gene transfer may happen and thus the question on the implications of recombinant DNA transfer has to be asked. It is obvious that the issue of the frequency of HGT is of minor importance. Even very rare events may have an ecological impact if the transferred gene increased the ecological fitness of the recipient organism. Hence, the genes encoded by the recombinant DNA pose a potential risk which should be the focus of biosafety considerations, rather than a natural phenomenon—horizontal gene transfer—itself.

When assessing the risk of the transfer of antibiotic resistance genes from transgenic plants to the autochthonous microflora, the question of the kind of the gene under consideration arises. If genetic determinants mediating resistance to a given antibiotic are widespread in natural bacterial communities, the potential transfer of the corresponding gene from transgenic plants to the soil bacterial community would not add something new to the bacterial gene pool. For example,

such extremely rare transfer events involving the *nptII* gene are negligible since resistance to kanamycin/neomycin is widespread in soil and phytosphere bacterial communities (Cole and Elkan, 1979; van Elsas and Pereira, 1986; Henschke and Schmidt, 1990; Smalla et al., 1993; Björklöf et al., 1995). Moreover, the probability that a potential recipient present in the phytosphere may acquire such a resistant determinant, e.g. located on a conjugative element seems to be much higher than the probability to receive the determinant from a transgenic plant. This conclusion can be drawn from the results of the above cited studies analysing gene transfer in the phytosphere. Although the *nptII* gene may be considered as a 'low risk marker', it seems questionable whether the presence of this or other antibiotic resistance genes in transgenic plants is necessary. Hence, effort should be put into the development of new strategies to remove antibiotic resistance genes from transgenic constructs.

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