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ENVIRONMENT DIRECTORATE
JOINT MEETING OF THE CHEMICALS COMMITTEE AND
THE WORKING PARTY ON CHEMICALS, PESTICIDES AND BIOTECHNOLOGY

GUIDANCE DOCUMENT ON HORIZONTAL GENE TRANSFER BETWEEN BACTERIA

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Guidance Document on Horizontal Gene Transfer between Bacteria

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FOREWORD

The OECD's Working Group on Harmonisation of Regulatory Oversight in Biotechnology decided at its first session, in June 1995, to focus its work on the development of ***consensus documents*** which are mutually acceptable among Member countries. These consensus documents contain information for use during the regulatory assessment of a particular product.

On reviewing a published consensus document and drafting other consensus documents on micro-organisms, the Working Group felt that these documents did not focus in a straightforward way on questions that are relevant to risk/safety assessment issues.

Responding to the concern, the Working Group decided to take an alternative approach, namely the development of ***guidance documents*** in the micro-organisms area which are intended to provide guidance on specific topics and issues that are relevant to risk/safety assessment in biotechnology. The Working Group also established a sub-working group that consists of experts on micro-organisms and focuses on projects related to the safety of environmental applications involving micro-organisms, especially bacteria. Subsequently, the sub-working group has developed several documents useful for environmental risk/safety assessment of micro-organisms within the Working Group.

This guidance document addresses a significant topic related to risk/safety assessment, horizontal gene transfer between bacteria. It is primarily intended for use by risk assessors, but may also be useful for applicants and other stakeholders in the regulatory process.

Germany served as lead countries in the preparation of this document, cooperating with the chair of the sub-working group. It has been revised on a number of occasions based on the input from other member countries.

This document is published on the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology of the OECD.

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1. Introduction

1. Horizontal gene transfer (HGT) [1] refers to the stable transfer of genetic material from one organism to another without reproduction. The significance of horizontal gene transfer was first recognised when evidence was found for 'infectious heredity' of multiple antibiotic resistance to pathogens (Watanabe, 1963). The assumed importance of HGT has changed several times (Doolittle *et al*., 2003) but there is general agreement now that HGT is a major, if not the dominant, force in bacterial evolution. Massive gene exchanges in completely sequenced genomes were discovered by deviant composition, anomalous phylogenetic distribution, great similarity of genes from distantly related species, and incongruent phylogenetic trees (Ochman *et al*., 2000; Koonin *et al*., 2001; Jain *et al*., 2002; Doolittle *et al*., 2003; Kurland *et al*., 2003; Philippe and Douady, 2003). There is also much evidence now for HGT by mobile genetic elements (MGEs) being an ongoing process that plays a primary role in the ecological adaptation of prokaryotes. Well documented is the example of the dissemination of antibiotic resistance genes by HGT that allowed bacterial populations to rapidly adapt to a strong selective pressure by agronomically and medically used antibiotics (Tschäpe, 1994; Witte, 1998; Mazel and Davies, 1999). MGEs shape bacterial genomes, promote intra-species variability and distribute genes between distantly related bacterial genera.

2. Horizontal gene transfer (HGT) between bacteria is driven by three major processes: transformation (the uptake of free DNA), transduction (gene transfer mediated by bacteriophages) and conjugation (gene transfer by means of plasmids or conjugative and integrated elements). These will be discussed in more detail below.

3. Mobile genetic elements (MGEs) such as plasmids, bacteriophages, integrative conjugative elements, transposons, IS (insertion sequence) elements, integrons, gene cassettes and genomic islands are the important vehicles in HGT. A brief summary of characteristic properties of MGEs is given in Table 1 (modified from Dobrindt *et al*., 2004). In many species a high proportion of horizontally transferred genes can be attributed to plasmid, phage or transposon-related sequences since remnants of MGEs are often found adjacent to genes identified as horizontally transferred in their complete genome sequences (Ochman *et al*., 2000; Brüssow *et al*., 2004; Gal-Mor and Finlay, 2006). MGEs are essential components that promote rapid adaptation to altered environmental conditions and as a consequence lead to bacterial diversification.

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[1] '*Horizontal gene transfer*' is synonymous with '*lateral gene transfer*' and results in unidirectional gene exchange (donor to recipient) between closely related or distantly related organisms; may be accompanied by expression of the introduced genetic material.
'*Transfer*' refers to translocation of genetic material into a cell, followed by stable integration into the recipient genome, including autonomously-replicating components of the genome (*e.g.* a plasmid, accessory chromosome or organelle chromosome). The transferred gene can be perpetuated in the offspring of the recipient organism.
'*Genetic material*' refers to any fraction of the genome (DNA or RNA), usually a gene or part thereof, which can include coding and/or non-coding sequences.
'*Organism*', as used here, includes cellular organism or replication competent virus.
'*Reproduction*' refers to the generation of offspring sexually, parasexually or asexually.
HGT can be differentiated from other types of gene transfer: (1) *Intentional gene transfer* – stable transmission of genes to a recipient organism through directed human intervention; (2) *Transient gene transfer* – transfer of genetic material to a recipient organism, intentionally or unintentionally, that is not perpetuated in the offspring; (3) *Intra-genomic gene transfer* – transfer of genetic material to a different location in the genome of the same organism (transposition); (4) *Vertical gene transfer* – transfer of genetic material from parent to offspring by reproduction, sexual or asexual. HGT contrasts with vertical gene transfer in that it can result in gene transfer between distantly related bacteria.

Table 1. Characteristics of mobile genetic elements (MGEs)

MGEs	Properties	Review
Plasmids	Circular or linear extrachromosomal replicons; self-transferable or mobilisable plasmids are vehicles for the transmission of genetic information between a broad or narrow range of species	Thomas, 2000; Thomas and Nielsen, 2005
Bacteriophages	Viruses that infect prokaryotes; can integrate into the host genome and then be vehicles for horizontal gene transfer	Canchaya *et al*., 2003
Integrative conjugative elements (ICE)	Self-transferable conjugative elements that integrate into the genome of new hosts like temperate bacteriophages; may promote the mobilisation of genomic islands by utilising conserved integration sites	Burrus and Waldor, 2004
Genomic islands	Large chromosomal regions acquired by horizontal transfer that are flanked by repeat structures and contain genes for chromosomal integration and excision	Dobrindt *et al*., 2004; Gal-Mor and Finlay, 2006
Transposable elements	Genetic elements that can move within or between replicons by action of their transposase; flanked by inverted repeats; transposons typically carry genes for antibiotic resistance or other phenotypes, while IS-elements code only for the transposase; multiple copies of the same IS-element promote genome plasticity by homologous recombination; IS*CR* (common region) s are a newly discovered class of transposable elements which mobilise DNA adjacent to their insertion site by rolling circle replication (those studied so far were closely associated with many antibiotic resistance genes and often located on conjugative plasmids)	Mahillon and Chandler, 1998 Toleman *et al*., 2006
Integrons	Genetic elements that capture promoterless gene cassettes into an attachment site downstream of a promoter by action of the integrase encoded on the integron; non-autonomous, but frequently associated with transposons and conjugative plasmids	Hall and Collis, 1995

MGEs play a significant role in HGT in three ways:

1) MGEs have evolved mechanisms that enhance the potential for gene transfer between organisms. For example, conjugative elements and viruses have evolved highly efficient mechanisms for the passage of genes into a recipient cell.

2) MGEs can alter the function of genes in the vicinity of the insertion in the host genome. These alterations can include disruption or inactivation of genes at the site of insertion. Conversely, insertional mutagenesis by MGEs can also result in benefits to the host such as provision of regulatory sequences, repair of double stranded DNA breaks, or genome restructuring and speciation.

3) HGT of MGEs can result in the transfer of additional genes through genetic piggy-backing. For example, MGEs are the primary vehicles for the spread of antibiotic genes, pathogenic determinants, and biodegradation pathways amongst bacteria (de la Cruz and Davies, 2000; Top and Springael, 2003; Smets and Barkay, 2005; Gal-Mor and Finlay, 2006; Larraín-Linton *et al*., 2006).

HGT has enabled bacterial populations to occupy entirely new niches (Burrus and Waldor, 2004).

4. Considering the plasticity of bacterial genomes, the aspect of horizontal gene transfer is of importance for biosafety evaluations of transgenic[2] micro-organisms. For transgenic micro-organisms the transfer of the transgenic DNA to other bacteria as well as the uptake of MGEs, which might change the

[2] In this document, the term 'transgenic' organisms is used interchangeably for 'genetically modified' as well as 'genetically engineered' organisms; considered under these terms are organisms that have been obtained by recombinant DNA methods.

characteristics of the released strain, are generally to be considered. Bacteria modified by means of genetic engineering as well as natural bacterial populations will exploit the horizontal gene pool to adapt to changing environmental conditions and together with gene loss and genetic alteration, the acquisition of horizontally acquired DNA will play an important role in their adaptive evolution (Dobrindt *et al*., 2004; Thomas and Nielsen, 2005; Gal-Mor and Finlay, 2006). Thus HGT needs to be understood as a natural process and major driving force of bacterial adaptability and diversity.

5. The intention of this document is to summarise the present state of knowledge on HGT between bacteria. The mechanisms of bacterial gene transfer and their occurrence under different environmental conditions will be briefly discussed, as well as implications for biotechnological applications.

2. Natural transformation

6. Natural transformation is generally understood as the uptake of free DNA by competent bacteria (Lorenz and Wackernagel, 1994; Dubnau, 1999, Chen and Dubnau, 2004). Natural competence is a genetically programmed physiological state permitting the efficient uptake of macromolecular DNA. Natural transformation is a tightly regulated process which requires elaborate machinery with more than a dozen of proteins involved. Transformability seems to be a property which is not shared by all isolates belonging to the same species, and transformation frequencies can vary up to four orders of magnitude among transformable isolates of a species (Sikorski *et al*., 2002; Maamar and Dubnau, 2005). The uptake of DNA can serve as a nutrient source, for DNA repair or as a source of genetic innovation (Dubnau, 1999). The uptake of DNA can be followed by integration into the bacterial genome by homologous recombination, homology-facilitated illegitimate recombination (de Vries and Wackernagel, 2002), or by forming an autonomously replicating element. The absence of homologous sequences or origins of replication were identified as major barriers to HGT by transformation (Thomas and Nielsen, 2005). Furthermore, spontaneous transformation or transformation by lightning (Cérémonie *et al.*, 2004, 2006) was also described for some bacteria which do not possess genes involved in natural competence. Natural transformation provides a mechanism of gene transfer that enables competent bacteria to generate genetic variability by making use of DNA present in their surroundings (Dubnau, 1999; Nielsen *et al*., 2000). Prerequisites for natural transformation are the availability of free DNA, the development of competence, and the uptake and stable integration or autonomous replication of the captured DNA. However, there is limited knowledge of how important natural transformation is in different environmental settings for the adaptability of bacteria. Transformation may be critical for the establishment, maintenance and gene transfer in bacterial biofilms (Molin and Tolker-Nielsen, 2003; Petersen *et al*., 2005). Two other aspects of natural transformation in the environment have been mainly studied: the persistence of free DNA and the ability of different bacterial species to become competent and take up free DNA under environmental conditions.

7. *Persistence of free DNA in soil* - Recent reports have shown that in spite of the ubiquitous occurrence of DNases high molecular weight free DNA could be detected in different environments. It is supposed that free DNA released from micro-organisms or decaying plant material can serve as a nutrient source or as a reservoir of genetic information for indigenous bacteria. Reports on the persistence of nucleic acids in non-sterile soil have been published (Blum *et al*., 1997; Nielsen *et al*., 1997a), and microbial activity was pinpointed as an important biotic factor affecting the persistence of free DNA in soil. Stimulated microbial activity often coincided with an increase in DNase activity in soil (Blum *et al*., 1997). Cell lysates of *Pseudomonas fluorescens*, *Burkholderia cepacia* and *Acinetobacter* spp. were available as a source of transforming DNA for *Acinetobacter* sp. populations in sterile and non-sterile soil for a few days, and Nielsen *et al*. (2000) showed that cell debris protected DNA from degradation in soil. Cell walls may play an important role in protecting DNA after cell death (Paget and Simonet, 1997). Long-term persistence up to two years of transgenic plant DNA was reported by Widmer *et al*. (1996, 1997),

Paget and Simonet (1997), and Gebhard and Smalla (1999) in microcosm and field studies. A more rapid break-down of transgenic DNA was observed at higher soil humidity and temperature. Both factors are supposed to contribute to a higher microbial activity in soil (Widmer *et al*., 1996; Blum *et al*., 1997).

8. Binding of DNA to rather different surfaces such as chemically purified mineral grains of sand, clay, non-purified mineral materials as well as humic substances has been reported (Khanna and Stotzky, 1992; Romanowski *et al*., 1992; Recorbet *et al*., 1993; Gallori *et al*., 1994; Lorenz and Wackernagel, 1994; Crecchio and Stotzky, 1998; Demanèche *et al*., 2001a). In the study of Demanèche *et al*. (2001a) plasmid DNA adsorbed on clay particles was found to be not completely degradable even at high nuclease concentrations. The adsorption of DNA seems to be a charge-dependent process and thus the rate and extent of adsorption of dissolved DNA to minerals depends largely on the type of mineral and the pH of the bulk phase, whereas the conformation and the molecular size of the DNA molecules have a minor effect (Lorenz and Wackernagel, 1994; Paget and Simonet, 1994). Since DNA can persist adsorbed on soil particles or protected in plant or bacterial cells, this DNA could be captured by competent bacteria colonising in close vicinity.

9. *Competence development* - Although it is supposed that natural competence is widespread among bacterial species (Lorenz and Wackernagel, 1994; Dubnau, 1999; Chen and Dubnau, 2004), the proportion of bacteria in natural settings which can become competent and the environmental conditions stimulating competence development is largely unknown. Only for a rather limited number of bacterial species have the natural transformation systems been studied in great detail (reviewed by Dubnau, 1999): *Bacillus subtilis, Streptococcus pneumoniae, Haemophilus influenzae, Neisseria gonorrhoeae, Acinetobacter* sp., *Pseudomonas stutzeri*, *Helicobacter pylori.* In the Gram-positive model bacteria studied, the first step in transformation is the binding of the double-stranded DNA to the cell, with no base sequence preference, followed by a fragmentation of the bound DNA. While the single strands are transported across the membrane the non-transported strand is degraded. The efficient uptake of DNA by the two Gram-negative model organisms *Haemophilus influenzae* and *Neisseria gonorrhoeae* require the presence of specific uptake sequences which are often found in the inverted repeats of the donor sequences. After binding the DNA becomes rapidly DNase resistant and is taken up after fragmentation. In addition to differences in the DNA uptake processes, bacteria do not exhibit the same efficiency to integrate the incoming DNA by heterologous recombination (Sikorski *et al*. 2002). The vast majority of studies on transformation in the context of biosafety research have been performed with strain *Acinetobacter* sp. BD413. Recently, the naturally transformable *Acinetobacter* sp. ADP1 strain and its derivative BD413 were shown to belong to the newly described species *Acinetobacter baylyi* (Vaneechoutte *et al.,* 2006). This strain can be efficiently transformed with DNA of different sources. Although the number of bacterial species for which natural transformation has been observed is growing, the majority of bacterial isolates in culture collections have not yet been tested to determine whether they can be transformed. Even more scarce are the data on natural transformation *in situ*. A few reports on the development of the competence state under environmental condition exist. Nielsen *et al*. (1997a, b) showed that the addition of nutrients can stimulate competence development of *Acinetobacter* sp. in bulk soil. Competence development was reported for the plant pathogen *Ralstonia solanacearum* and the co-inoculated *Acinetobacter* sp. BD413 when colonising tobacco plants. Even more striking is the observation of natural transformation in soil with two soil isolates *Pseudomonas fluorescens* and *Agrobacterium tumefaciens* (Demanèche *et al*., 2001b). However, transformation frequencies were very low and often not reproducible. Marker rescue based on restoration of deleted *nptII* was also used to study transfer of bacterial or transgenic plant DNA in *Streptococcus gordonii*. While *in vitro* transformation could be achieved, no marker rescue was observed in gnotobiotic rats, presumably due to the absence of competence-stimulating factors like serum proteins (Kharazmi *et al*., 2003). A peptide-pheromone system which controls genetic competence in *Streptococcus mutans* functions optimally when cells are living in actively growing biofilms (Li *et al*., 2001). Biofilms seemed also to facilitate natural transformation of *Acinetobacter* sp. BD413 and did not offer a barrier against

effective natural transformation (Hendrickx *et al*., 2003). Chitin, an abundant surface particularly in aquatic habitats, induces natural competence in *Vibrio cholerae* (Meibom *et al*., 2005).

3. Transduction

10. Transduction is a mechanism of DNA acquisition by which non-viral DNA can be transferred from an infected host bacterium to a new host via infectious or non-infectious virus particles. Host DNA is mistakenly packaged into the empty phage head when the phage particle is produced. Defective phage particles which are released from lysed host cells can adsorb to new host cells and deliver the DNA carried in the capsid into the new host (Brüssow *et al*., 2004). However, the incomplete phage genome does not allow progeny phage production. The injected bacterial DNA can be integrated into the recipient genome using mechanisms that generally require the presence of homologous DNA sequences or specialised integrases. Based on the phenotype of the bacteriophage mediating the genetic transfer, generalised or specialised transduction are distinguished. While generalised transducing phages can be either temperate or virulent phages which carry any kind of host DNA regardless of location, specialised transducing phages are temperate phages and transfer only specific genes located close to the phage integration site.

11. An important aspect of prophage acquisition is that the bacterial hosts can gain fitness advantages, *e.g.,* immunity against lytic infections. Furthermore, phages play an important role in the emergence of bacterial pathogens. Many pathogenicity determinants (toxins) have been acquired via phages, *e.g.,* by *Corynebacterium diphtheria*, *Clostridium botulinum*, *Streptococcus pyogenes*, *Staphylococcus aureus* and Shiga toxin producing *E. coli* (reviewed by Brüssow *et al*., 2004). Pathogenicity islands (PAI) which are defined as large genomic islands that carry one or more virulence gene and occur in the pathogenic variants of a species but not or less frequently in the non-pathogenic strains often evolved from lysogenic bacteriophages (Hacker *et al*., 2003; Dobrindt *et al*., 2004; Brzuszkiewicz *et al.*, 2006). Forty of 56 sequenced bacterial genomes recently reviewed by Canchaya *et al*. (2003) contain prophage sequences, which are viral nucleic acids that have been incorporated into the bacterial genome, exceeding 10 kb in length.

12. Although most bacteriophages infect only a narrow range of hosts, this mechanism of gene transfer has the advantage that transducing phages can be rather persistent under environmental conditions, do not require cell-cell contact, and DNA in transducing phage particles is protected (Wommack and Colwell, 2000). Marine environments are probably a major setting for virus mediated gene transfer between bacteria, where there is an estimated abundance of greater than 10^{29} virus particles (Hendrix *et al*., 1999; Weinbauer and Rassoulzadegan, 2004). For example, virus mediated gene transfer frequencies of around 10^{-8} have been reported from the Tampa Bay estuary, corresponding to around 3.6×10^{11} HGT events each day in the estuary (Jiang and Paul, 1998).

4. Conjugation

13. The process whereby a DNA molecule is transferred from a donor to a recipient cell via a specialised protein complex is termed conjugation. Conjugation requires close physical contact between donor and recipient populations and the DNA (plasmids or conjugative transposons) is transferred via the so-called conjugation apparatus (Zechner *et al*., 2000). Although common mechanistic principles are shared by most of the conjugative system, *e.g.*, the synthesis of conjugative pili, there is a remarkable diversity of conjugative systems in Gram-negative and Gram-positive bacteria. Conjugative transfer requires the activity of the mating pair formation complex (Mpf) and of the DNA transfer and replication system (Dtr). While the former is responsible for making the contact between donor and recipient, the latter provides the functions for processing of DNA for transport. The plasmid encoded pili have different

properties, *e.g.*, pili encoded by the so-called broad host range plasmids IncP-1, IncN or IncW are short and rigid while other plasmids such as IncF encode for flexible pili. Pilus properties are supposed to determine in which environmental niches the respective plasmid types transfer efficiently. Interestingly, plasmid encoded Mpf complexes are phylogenetically highly related to bacterial transport systems for proteins, the so-called type IV-secretion pathways. However, the question of whether the DNA traverses via the Mpf complex remains still to be experimentally proven.

14. A critical property of plasmids is their host range since this determines how far they can cause spread of antibiotic resistance or other phenotypes without physical recombination with the DNA of the new host. HGT can only affect bacteria that readily exchange genes. Bacterial genome comparisons indicated that members of 'exchange communities' have a tendency to be similar in factors like genome size, genome G/C composition, carbon utilisation, and oxygen tolerance (Jain *et al*., 2003). Host range in general appears to be limited by the interaction of the plasmid and its gene products with host enzymatic machinery. Host range is not an all or nothing property, but in the environment, on the one hand certain species or strains are preferred among the potential hosts (Heuer *et al*., 2007), on the other hand, MGEs are typically not fixed globally but persist in patches of local subpopulations (Berg and Kurland, 2002). This supports the notion that conjugative plasmids and the MGEs carried by them spread among a limited number of host cells and thus contribute to diversification of populations.

15. IncP-4 (group 4 from *Pseudomonas* species corresponding to IncQ) plasmids appear to be able to replicate in all Gram-negative bacteria and even some Gram-positive bacteria. This promiscuity may be due to their encoding three replication proteins and thus being largely independent of the replication machinery of their host. IncP-1 plasmids synthesise two related replication proteins encoded by overlapping genes and are able to replicate and be stably maintained in all Gram-negative bacteria but not Gram-positive bacteria. However, recently a cultivation-independent examination of the host range of IncP-1 plasmid in the rhizosphere of barley revealed an extremely broad host range and detected the IncP-1 plasmid (pKJK10) in *Arthrobacter* sp., a member of the *Actinobacteria* (Musovic *et al.,* 2006). Plasmids with a broad host range often appear to have lost restriction sites by point mutation and selection enzymes in the strain to which they are transferring. In addition they may carry anti-restriction systems that minimise the effect of cleavage by special nucleases that protect many bacteria from invasion by foreign DNA. In summary, the host range of a plasmid may be determined by a range of small interactions. A narrow host range plasmid is no guarantee of containment in use of transgenic organisms. Point mutations may well extend the host range (Maestro *et al*., 2003), or environmental hosts may adapt to the plasmid (Heuer *et al*., 2007).

5. Other mechanisms of HGT

16. In addition to conjugation, transformation and transduction, other less well recognised mechanisms of DNA uptake occur in nature but their significance is uncertain. One such mechanism is *Vesicle-mediated translocation* whereby a range of Gram-negative bacteria such as *Neisseria gonorrheae*, *E. coli* and *Pseudomonas aeruginosa* can bud off vesicle structures that contain genetic material (*e.g.*, antibiotic resistance and virulence genes), which then fuse with another bacterium (Dorward *et al*., 1989; Yaron *et al*., 2000). Another mechanism of HGT involves *Pseudovirus particles*, in which some bacteria have genes that encode proteins capable of forming virus-like particles (gene transfer agent), which can trap random fragments of the genome (about 4,400 – 13,600 base pairs) and transmit them to a second bacterium (Marrs, 1974; Dykhuizen and Baranton, 2001; Lang and Beatty, 2001).

6. Prevalence of mobile genetic elements in bacterial communities from different environmental habitats

17. The use of genomic approaches to study the prevalence of MGEs resident in environmental bacteria has revealed a large diversity. The presence of MGEs in environmental bacteria is more the rule than the exception. Depending on the isolation procedure, different plasmids, with diverse characteristics with respect to Inc group, host range, avidity to transfer and the type of accessory genes present, can be obtained.

18. Surveys on the presence of plasmids in bacteria isolated from a wide range of environments have been performed and reveal that a considerable proportion of bacteria from different environments carried plasmids. Approximately 18% of bacterial isolates from the phytosphere of sugar beets were found to contain plasmids (Powell *et al*., 1993) and a large proportion of these plasmids were able to mobilise non-self-transferable but mobilisable IncQ plasmids (Bailey *et al*., 1994). To whatever extent environmental samples have been used, recipients functioning as a genetic sink and introduced under laboratory or *in situ* conditions have acquired MGEs conferring selectable traits such as mercury or antibiotic resistance (Smalla and Sobecky, 2002). In several of the studies increased transfer frequencies were observed when the environmental samples were stressed with pollutants. Mercury resistance was also used as an effective selective marker to exogenously isolate self-transferable Hg^r plasmids from river epilithon (Bale *et al*., 1988), the phylloplane and rhizosphere of different crops or sediments in Gram-negative recipients (Lilley *et al*., 1994, 1996; Smit *et al*., 1998; Schneiker *et al*., 2001; Smalla *et al*., 2006). *In situ* acquisition of mercury resistance plasmids by a transgenic *Pseudomonas fluorescens* SBW25EeZY6KX colonising the phytosphere of sugar beets observed under field conditions in two consecutive years confirmed this result (Lilley and Bailey, 1997a). Transconjugants which acquired Hg^r plasmids were isolated from the rhizosphere and the phyllosphere only at a certain time of plant development. Capturing of degradative genes resident on MGEs has also been demonstrated (Top *et al*., 1995, 1996). Self-transferable plasmids conferring resistance towards a range of antibiotics were captured from activated sludge, sewage or animal manures in green fluorescent protein (*gfp)*-labelled Gram-negative recipients (Dröge *et al*., 2000, Smalla *et al*., 2000; Heuer *et al*., 2002, Van Overbeek *et al*., 2002; Heuer and Smalla, 2007). Many of the exogenously isolated MGEs were shown to belong to the IncP-1 group (Heuer *et al*., 2002, 2004; Schlüter *et al*., 2003; Smalla *et al*., 2006). Inc-P1 plasmids transfer to and replicate in a wide range of Gram-negative bacterial hosts. Sequencing of a number of IncP-1 plasmids isolated from different sources showed that they all contain blocks of accessory DNA, *e.g.*, transposons, integrated between backbone modules coding for replication, maintenance and transfer of the plasmid. Transferable antibiotic resistances could be isolated from most of the environments analysed (Heuer *et al*., 2002; Van Overbeek *et al*., 2002). Using this approach not only could conjugative plasmids be isolated from manure bacteria but also mobilisable IncQ-like plasmids indicating the presence of mobilising plasmids in piggery manure (Smalla *et al*., 2000). Mobilising plasmids were isolated by Van Elsas *et al*. (1998) when bacterial communities obtained from the rhizosphere of young wheat plants served as donor in triparental matings. Plasmid pIPO2 was isolated in *R. eutropha* based on its mobilising capacity. Replicon typing and sequencing of the complete plasmid (Tauch *et al*., 2002) revealed that this cryptic plasmid of approx. 45 kbp was not related to any of the known broad host range (BHR) plasmids except to plasmid pSB102 (Schneiker *et al*., 2001). The role of these plasmids for their plant-associated bacterial host remains unknown.

7. Factors affecting transfer efficiency of MGEs

19. The majority of natural environments - soils and aquatic systems - are restricted by the abundance of resources for microbial growth, which can severely limit population densities and activity. This, in turn, restricts those microbial processes that are dependent on density and activity, such as all HGT mechanisms (Van Elsas *et al*., 2000; Timms-Wilson *et al*., 2001). However, particular sites in these natural

habitats, mostly related to soil or plant surfaces or surfaces in aquatic environments, have been shown to provide conditions for bacterial colonisation, mixing and activity, resulting in the occurrence of locally-enhanced densities of active cells. Transformation and conjugation processes were also shown to contribute to biofilm formation (Ghigo, 2001; Reisner *et al.*, 2006). These sites are often conducive to HGT processes, and are regarded as "hot" spots for bacterial gene transfer activity. Key abiotic and biotic factors that affect the extent of HGT in hot spots in natural settings have been reviewed, but quantitative prediction of natural HGT is still not possible presumably due to interactive effects between the different factors (Van Elsas *et al*., 2000; Van Elsas and Bailey, 2002).

20. Soil and phytosphere - The presence of large surfaces composed of mineral and organic phases in soil plays a key role in determining the physiological status of soil-dwelling bacterial cells. Soil is heterogeneous with regards to the distribution of gaseous, liquid or solid compounds (Stotzky, 1997). Clay-organic matter complexes are important sites for soil micro-organisms, due to their negatively-charged surfaces and enhanced nutrient availability. Availability of water in soil is a second important factor driving microbial activity. In bulk soil, bacterial cells occur mainly adsorbed to surfaces, which often results in micro-colonies that are refractory to movement or to contact with cells at different locations. Hence, most bacterial cells in soil can interact only with other bacteria in their immediate vicinity. Conditions that apply locally, *i.e.,* at the level of the site where bacterial cells are localised, will affect the cells and their involvement in HGT. In spite of the grossly nutrient-poor status of soil (Van Elsas *et al*., 2000; Timms-Wilson *et al*., 2001), nutrients can become concentrated in hot spots, primarily plant phytospheres, decaying organic material of animal or plant origin, and guts of soil animals like earthworms (Daane *et al*., 1996; Thimm *et al*., 2001) and Collembola (Hoffmann *et al*., 1998). The rhizosphere of many plants represents a region in soil with a (transient) high availability of organic carbon and potentially also N, P and S. Moreover, water flow in soil induced by plant roots may enhance bacterial movement. Both mechanisms promote cellular activities and cell-to-cell contacts. Recently, Mølbak *et al*. (2007) showed plant-dependent cell densities and distribution of donors and transconjugants in the rhizosphere of pea and barley. Thus exudation and root growth seem to be key parameters controlling plasmid transfer in the rhizosphere (Mølbak *et al.*, 2007).

21. Moreover, aboveground plant parts (the phyllosphere) can also provide nutrient-rich surfaces, resulting in similar hot spots (Björklöff *et al*., 1995). Further, as mentioned above, the guts of a range of soil animals represent another class of hot spots, as the mixing of cells and MGEs is enhanced, cells are activated and cell-to-cell contacts are stimulated (Thimm *et al*., 2001; Hoffmann *et al*., 1998). Finally, the importance for HGT processes of easily-available substrate in soil, such as those provided by manure, has been indicated by Götz and Smalla (1997) and Heuer and Smalla (2007). Thus, soil, on the one hand, poses physical barriers to cell-to-cell contacts and nutritional limitations, whereas, on the other hand, nutrient up-shifts and alleviation of translocation or contact barriers may be found in soil hot spots. HGT rates in soil are certainly affected by the combination of these phenomena.

22. Selective pressure can be key in exacerbating the impact of gene transfer processes. Effects of selective pressure are most easily seen in cases in which the MGEs transferred confer some type of selective (growth) advantage to their hosts. Top *et al*. (2002) recently reviewed the issue of selection acting on gene transfer in soils. Several studies showed strong effects of selection. Transfer of a catabolic 2,4-dichloropropionate (DCPA) degrading plasmid from an *Alcaligenes xylosoxidans* donor to members of the indigenous community in soil, was only seen in soil treated with DCPA. When *Enterobacter agglomerans* carrying the self-transmissible biphenyl-degradative plasmid RP4::Tn*4371* was introduced as a (non-expressing) donor into soil with or without added biphenyl the introduced donor strain declined to extinction very quickly while indigenous transconjugants belonging to the genera *Pseudomonas* and *Comamonas* appeared. Again, transconjugants were only detected in soil that had received biphenyl. Furthermore, the transfer of the herbicide 2,4-D degradative plasmid pJP4 from *Ralstonia eutropha* JMP134 to *Variovorax paradoxus* was only detectable in soil in the presence of high levels of 2,4-D, and

the transfer frequency of pJP4 to indigenous *Pseudomonas* and *Burkholderia* spp. increased as 2,4-D concentrations increased. Proliferation of the new transconjugants formed in soil was noted, which included representatives of *Burkholderia graminis*, *B. caribensis* and *R. eutropha*. The acquisition of novel pathways by HGT resulted in the adaptation of the indigenous bacterial communities to utilise the xenobiotic compounds as sources of nutrients. Evidence has also been provided for a direct role for genetic recombination in the adaptation of bacterial aquifer communities to chlorobenzenes (Van der Meer *et al*., 1998). These and related studies (Herron *et al*., 1998) demonstrated the central role that HGT plays in the adaptation of bacterial communities to changing resources and environmental pressures, such as novel substrate utilisation, antibiotic resistance and toxin production.

23. *Aquatic habitats and biofilm* - Aquatic systems contain specific structured (micro)habitats that may represent hot spots for HGT. Conceptually, aquatic systems can be divided in (1) the free (bulk) water phase, (2) the colonisable suspended matter, (3) sediment or sewage, (4) stones and other surfaces that carry biofilms (called epilithon), and (5) aquatic animals. These habitats offer very different conditions to their bacterial inhabitants. The presence of nutrients as well as colonisable surfaces is particularly important as such sites are known to support large densities of metabolically-active micro-organisms (Hill *et al*., 1994). In contrast, bulk water can be a nutrient-poor environment which may induce a (starvation) stress response in bacterial cells. Environments such as sewers, with high inputs of organic matter, form an obvious exception to this generalisation. On the other hand, suspended particles of varying sizes, as well as sediment and stone surfaces, represent nutrient-rich, more hospitable habitats that support microbial communities. Further, sediments, which are often rich in organic material, typically support bacterial populations exceeding those found in bulk water by several orders of magnitude (Van Elsas *et al*., 2000). Biologically diverse and metabolically active communities can also be found in the epilithon of stones in rivers or lakes (Hill *et al*., 1994; Van Elsas *et al*., 2000) and within other biofilms that form at solid/water interfaces. Micro-organisms within the epilithon are components of the extensive polysaccharide matrix which protects cells and adsorbs dissolved and particulate organic matter from the overlying water. Similar biofilms can be found in the percolating filter beds used in sewage treatment processes (Gray, 1992). Finally, a range of aquatic animals provide internal and external surfaces that are colonised by varied micro-organisms.

24. As bacterial hosts accumulate at surfaces where nutrients are concentrated, their distribution in aquatic systems is not even. The bulk water phase may contain relatively few bacterial cells that are able to participate in HGT processes. On the other hand, aquatic systems tend to provide excellent possibilities for mixing of bacterial cells and MGEs, and thus, for cell-to-cell and cell-to-MGE contacts. These contacts occur mainly in biofilms at surfaces. The tendency of bacterial cells to stick to suspended particles, sediment or stones (epilithon) in aquatic systems may lead to the development of separate communities. Nevertheless, given the capability of many bacteria to occur in either sessile forms in micro-colonies or biofilms, or in motile forms, and thus to potentially connect spatially-separated biofilms, aquatic habitats provide important sites for cell-to-cell contacts resulting in HGT between bacteria (Van Elsas *et al*., 2000). Using both microcosm and *in situ* experiments, HGT between bacterial hosts has been shown to occur in drinking water, river water and epilithon (Hill *et al*., 1994, 1996), lake water, seawater, marine sediment and wastewater (Van Elsas *et al*., 2000). HGT thus appears to be a common process in aquatic environments, particularly in specific niches where nutrients are more abundant.

25. *Animal ecosystems* – Much evidence was found for HGT of antibiotic resistance genes in the intestines of humans, farm animals, and insects, as reviewed by Davison (1999). Transfer of antibiotic resistance genes in the intestines was shown between a variety of Gram-positive and Gram-negative bacteria. Identical nucleotide sequences of resistance genes (*e.g., tetM*, *tetQ*) in bacteria from antibiotic-treated farm animals and humans suggested transfer of these genes between these habitats, raising the possibility of acquisition of resistances by human pathogens through the use of antibiotics as additives in agricultural animal feed. In a recent study, it was shown in the intestine of mice that bacteriostatic

compounds like tetracycline could increase spread and establishment of transconjugants which acquired a resistance conferring plasmid (Licht *et al*., 2003). Thus, the use of antibiotics may not only select for resistant populations but also enhance the formation of new resistant strains by HGT. The loss of IncP-1 plasmids from *Escherichia coli* cells colonising the gastrointestinal tract of germ-free rats was shown to be counteracted by the plasmid's ability to conjugate (Bahl *et al*., 2007).

8. Barriers to HGT

26. Although HGT can increase genetic diversity and promote the spread of novel adaptations, it can also result in excess genetic baggage and the import of deleterious genes. Therefore, organisms possess a number of physical, biochemical and genetic barriers to restrict the frequency of HGT (Kurland, 2005; Matic *et al*., 1996; Nielsen, 1998). Some of the barriers to HGT include the physical integrity of the cell; restriction-modification systems that recognise and hydrolyse foreign gene sequences; requirements for self-recognition sequences (*e.g.*, the genomes of *Haemophilus* and *Neisseria* have multiple copies of short sequences required for recognition and uptake by transformation); sequence specificity for integration into the recipient genome by homologous recombination; presence of inappropriate regulatory signals; nucleotide composition adaptations for optimised gene expression; mismatch repair systems; and natural selection. In general, the stringency of barriers to HGT increases proportionally with genetic distance. Consequently, the frequency of HGT is much greater within species than between unrelated or distantly related species.

9. Survival of MGEs

27. MGEs are generally agreed to add some, however small, burden to their host, although adaptation can occur to minimise this impact. This burden can be metabolic, *i.e.*, the need to copy and express extra genes, resulting in a change of phenotypic properties, for example, a change in the cell surface that is a disadvantage in specific circumstances, *e.g.*, when bacterial viruses are present. Thus it is generally agreed that the prevalence of plasmids must mean that they can be of benefit to bacteria to compensate for the burden they represent. The way they do this may not be identical for all plasmids. A small, high copy number plasmid and a large, self-transmissible plasmid may benefit its hosts in different ways. However, the broadest view is that MGEs increase the chance of new strains arising with novel or increased selective advantages over their neighbours. Since most naturally occurring plasmids can transfer between strains either by conjugation, transduction or transformation the autonomous replication ability of plasmids removes the need of a gene to integrate by recombination in to the chromosome of a new host. High copy number plasmids can also modulate phenotype by changing gene dosage as well as increasing mutation frequency. Thus MGEs carry niche traits and survive because they allow their new host to exploit the niche so long as they acquire the MGEs. If no other host presents itself to benefit from the properties that the MGEs can confer then in the long term strains in which the advantageous genes carried by the MGEs have integrated into the chromosome may arise and dominate. Thus MGEs survive because microbial communities, and their environments, are continually changing so that the variability that an MGE allows increases the speed at which adapted strains arise and the adapted strains carry the MGE and propagate it faster. Thus MGEs that increase adaptability evolve and will survive at the expense of those that do not.

10. Mobile genetic elements as tools for biotechnology

28. The study of degradative pathways is important both from environmental and evolutionary viewpoints. Natural gene exchange in bacterial communities in the environment is an important mechanism that allows the bacteria to acquire new genetic information and thus evolve abilities to degrade persistent chemicals. It has become clear that there is much more diversity among certain catabolic genes

and the mobile elements they reside on than we know so far. The source of genetic information for degradative pathways is still largely undiscovered. Plasmids encoding degradation of xenobiotic, often chlorinated compounds, seem to belong mainly to the IncP1 group. These IncP1 plasmids are the most promiscuous (or broad host range, BHR) self-transmissible plasmids characterised to date. If this correlation is true, it could suggest that recent bacterial adaptation to xenobiotics is promoted by plasmid promiscuity. A variety of plasmids involved in chloroaniline degradation have been described (Boon *et al*., 2001; Dejonghe *et al*., 2002). In addition to progress in insights in the role of BHR plasmids in metabolic pathway evolution, MGEs other than plasmids have been shown to carry catabolic genes and to be responsible for their lateral exchange, resulting in the assembly of new pathways. A new transposable element that codes for the degradation of biphenyl and 4-chlorobiphenyl was recently described (Merlin *et al*., 1999) and detected in several PCB degrading bacteria isolated from various environments (Springael *et al*., 2001). These recent findings strongly suggest that such MGEs play a very important role in the dissemination of degradative genes among bacteria, and thus in the natural construction of new degradative pathways. It is of great importance to better understand the underlying rules and mechanisms of these means of gene exchange, since they lead to accelerated removal of unwanted contaminants in natural environments.

29. Bio-augmentation can be defined as the addition of micro-organisms to a site in order to accelerate biodegradation of pollutants. This has been tested in several laboratory microcosm experiments, but in many cases the introduced bacteria have failed to degrade the pollutants due to their poor survival or low activity in their new environment. This may be due to abiotic and biotic stresses that are not encountered in the usual laboratory environment they have been maintained in for short to very long periods. An alternative approach involves the introduction and subsequent horizontal transfer of appropriate plasmid-borne catabolic genes into well-established and competitive indigenous bacterial populations. In this case, the survival of the introduced donor strain is no longer needed once the catabolic genes are transferred and expressed in the indigenous bacteria.

30. After inoculation with the 3-chlorobenzoate (3CBA) degrader *Pseudomonas putida* BN10 in a membrane biofilm reactor treating 3CBA containing synthetic wastewater, transfer of its mobile chlorocatechol catabolic genes (*clc*) to various bacterial populations was observed (Springael *et al*., 2002). Since the original inoculum strain always disappeared, the new 3CBA degraders seemed to out-compete the inoculum. Similar observations were made in different wastewater treatment systems inoculated with the same *clc*-element (Springael *et al*., 2002). These results show that treatment of wastewater contaminated with persistent pollutants can be improved by inoculating and spreading the necessary mobile genetic elements. Furthermore, it was demonstrated that bio-augmentation of soils contaminated with the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is possible via inoculation and subsequent transfer of a degradative self-transmissible plasmid (Dejonghe *et al*., 2000). The transfer of two different 2,4-D degradative plasmids to the indigenous bacteria of the A- and B-horizon of an agricultural soil was shown to positively affect the biodegradation of 2,4-D. This was most striking in the B-horizon without nutrient amendment, where no 2,4-D degradation occurred in the non-inoculated control soil during at least 89 days, while inoculation and subsequent plasmid transfer resulted in complete degradation of 2,4-D within 19 days. Overall, this work clearly demonstrates that bio-augmentation can constitute an effective strategy for clean-up of soils which are poor in nutrients and microbial activity.

31. As there are very few reports on bio-augmentation mediated by MGEs in soils and wastewater treatment reactors, the results summarised above suggest that natural MGEs that carry degradative genes could indeed be useful as tools in environmental biotechnology.

32. Since many bacterial species being used in biotechnological applications of various kinds are very distinct from *E. coli*, the cloning tools developed for *E. coli* often do not work for these organisms.

Therefore new plasmid vectors, reporter and marker gene constructs, and other MGEs have been developed.

33. Overall it is clear that MGEs are not just interesting objects to be studied from an evolutionary viewpoint, they can also clearly contribute to solving problems in society. One is environmental pollution, but many other obstacles that can only be overcome realistically by means of biotechnology will continue to need the development of new vectors and other MGEs to optimise micro-organisms for various biotechnological processes.

11. Tools to study the horizontal gene pool

11.1. Endogenous isolation

34. The traditional approach to analyse the presence of plasmids in bacteria is the so-called endogenous plasmid isolation technique which requires culturability of the plasmid host. Pure cultures of bacteria isolated either on non-selective media or on media supplemented with antibiotics, heavy metals or xenobiotics are subsequently screened for the presence of plasmids by extraction of plasmid DNA. Surveys on the presence of plasmids in bacteria from a wide range of environments have been performed and revealed that a considerable proportion of bacteria from different environments contain plasmids. Determination of plasmid-encoded traits requires curing or subsequent transfer of the plasmid to a well characterised host.

35. With the use of endogenous plasmid isolation, only plasmids present in culturable bacteria are accessible. The advantage of the endogenous plasmid isolation approach is that the plasmid host is known. Based on the characteristics of the plasmid host, prediction can be made on the occurrence and fate of the plasmid in different environmental niches. A clear limitation of the endogenous plasmid isolation technique is the dependence on culturability of the plasmid host. Another disadvantage of endogenous plasmid isolations is that nothing is known about the relevance of such plasmids to *in situ* gene transfer processes (Smalla *et al*., 2000; Smalla and Sobecky, 2002). Plasmids with a high transfer potential might be overlooked when their hosts are less dominant or belong to the majority of bacteria which are not accessible by the cultivation techniques used.

11.2. Exogenous isolation

36. The exogenous isolations are performed either in a biparental or triparental mating and MGEs are recovered directly from the bacterial fraction of the environmental sample in recipient cells. In a biparental mating the donor (bacterial fraction) is mixed for mating purposes with a recipient (Bale *et al*., 1988). The recipient needs an appropriate selectable marker which allows counter-selection against the donor. Due to the commonly low levels of natural background, rifampicin resistant recipients have been used frequently for matings. Recipients which have acquired a plasmid coding for an antibiotic or heavy metal resistance are obtained by plating on media supplemented with rifampicin and antibiotics or heavy metals (Hill *et al*., 1996; Smalla *et al*., 2000; Heuer *et al*., 2002; Smalla *et al*., 2006; Heuer and Smalla, 2007). Mating conditions and recipients chosen will strongly affect the range of mobile elements obtained. Exogenous plasmid isolations have been applied to retrieve biodegradative genes borne on plasmids (Top *et al*., 1995). *In situ* acquisition of mercury resistance plasmids by a transgenic *Pseudomonas fluorescens* colonising the phytosphere of sugar beets was demonstrated under field conditions (Lilley *et al*., 1994, 1996; Lilley and Bailey, 1997a).

37. A variety of recipient strains that were rifampicin resistant and/or tagged with a marker gene such as *gfp* or luciferase (*luc*) has been successfully applied to retrieve plasmids from different environments, *e.g.*, *Pseudomonas putida*, *Pseudomonas fluorescens*, *Escherichia coli*, *Enterobacter cloacae*, *Ralstonia eutropha, Sinorhizobium meliloti* and *Agrobacterium tumefaciens*. However, until now exogenous isolation of plasmids into Gram-positive strains (either lab strains or isolates from the rhizosphere) has not yet been reported. The biparental exogenous isolation allows plasmids from environmental samples to be obtained independently from the culturability of the original host. Isolations of MGEs are based on their transfer efficiency under the mating conditions chosen, as well as their ability to replicate and express selectable marker genes in the recipient background. Traits newly acquired by the host are easily identified. A major disadvantage is that the original plasmid host remains unknown. While the biparental approach relies on the expression of selectable markers, the isolation of MGEs in triparental matings is solely based on their ability to mobilise small mobilisable plasmids carrying selectable markers into a new recipient (Hill *et al*., 1992; Van Elsas *et al*., 1998; Smalla *et al*., 2000). Using this methodology gene mobilising capacity was found in most environments analysed.

11.3. Polymerase Chain Reaction (PCR)-based detection of MGEs in directly extracted DNA

38. Many efficient methods to directly extract nucleic acids from various environmental samples are now available (Van Elsas *et al*., 2000). Two general approaches are used to extract nucleic acids from environmental samples: (I) Cell lysis within the environmental matrix or (II) cell lysis after recovery of the bacterial fraction from the environmental sample. To obtain DNA from environmental samples that are representative of the microbial community, the efficient lysis of diverse cells and spores is critical. Furthermore, co-extracted humic acids, which might inhibit the PCR amplification, need to be removed. The application of MGEs-specific primers to total community DNA can greatly facilitate the screening of and comparison of horizontal pools between different environments for the presence of MGEs such as transposons, plasmid groups or gene cassettes (Götz *et al*., 1996; Smalla *et al*., 2006; Heuer and Smalla, 2007). The growing sequence database for MGEs facilitates the design of primers specific for their detection. Primer specificity can be predicted based on sequence comparisons and should be confirmed experimentally. The PCR-based approach allows the detection of various MGEs independently from the culturability of their hosts, from the presence and expression of selectable markers, and from their ability to transfer to, and replicate in, a new recipient. MGE-specific sequences can be detected by PCR amplification even though they occur only in a minor fraction of the population. However, the main advantage of this approach is that large sample numbers can be analysed, making extensive screening programmes for a variety of environments more feasible. The obvious limitation is that information regarding the nature of the host(s) containing these various MGEs is not obtained.

11.4. New tools to study **in situ** *transfer processes*

39. Traditional studies on gene transfer in the environment have relied on cultivation-based techniques, by which donor, recipient and transconjugant, transductant or transformant colonies have been detected following their dislodgement from the environmental setting. A key factor has been the ability to select for donor, recipient and transconjugant cells using a combination of appropriate markers, including antibiotic resistance (Akkermans *et al*., 1995; Timms-Wilson *et al*., 2001). These approaches are now complemented with molecular methods such as PCR typing, DNA:DNA hybridisation, and sequencing, applied directly to habitat-derived DNA/RNA (Akkermans *et al*., 1995; Götz *et al*., 1996; Smalla *et al*, 2000) and to bacterial isolates. A major objective is the identification of specific sequences and functions relevant to HGT and the persistence of MGEs in response to environmental conditions. Also, reverse transcription (RT)-PCR analysis of environmental mRNA followed by hybridisation (micro-arrays), should facilitate the study of the expression of specific plasmid, phage, transposon or host genes in relation to

HGT in the natural environment. Thus, the distribution and activity of genes and transcripts can be determined to identify processes associated with the interaction between MGEs, bacteria and the environment. By contrast, other non-disruptive approaches exploit the potential of fluorescent markers, such as green fluorescent protein (*gfp*), for studying the transfer of plasmids (Christensen *et al*., 1996, 1998; Dahlberg *et al*. 1998a, 1998b; Sørensen *et al*., 2003, 2005). In particular, *in situ* monitoring of plasmid transfer and microbial community physiology in structured microbial communities (through fluorescent reporter systems and confocal laser scanning microscopy) has provided a greater understanding of these complex processes (Christensen *et al*., 1998; Heydorn *et al*., 2000). Using monitoring of expression of unstable fluorescent reporter proteins introduced into chromosomes or on MGEs with assembled biofilm communities, plasmid transfer and cellular activity could be directly recorded (Andersen *et al*., 1998). Furthermore, using fluorescence-labeled reporter plasmids HGT can be detected and quantified independent from cultivation techniques by flow cytometry (Musovic *et al*., 2006). These *in situ* observations illustrate the advances in our knowledge base beyond earlier efforts that were largely system-disruptive (Van Elsas *et al*., 2000). For example, the use of a donor containing a plasmid carrying a *gfp* gene repressed for expression in the donor facilitates the screening for plasmid transfer to nonculturables in which *gfp* is expressed. Recently, *in vivo* expression technology (IVET) was made available to studies on plasmid gene expression in soil and the phytosphere (Bailey *et al*., 2001). Using this method, plasmids can be shown to carry genes that are uniquely expressed in the phytosphere at different periods of colonisation during plant growth. These genes are distinct from those similarly expressed by the host bacteria and share little homology with database sequences. This supports previous data that plasmids can provide periodic fitness advantages to their hosts (Lilley and Bailey, 1997b). The challenge is to identify the phenotypes of ecologically significant genes and the contribution they make to host ecology and evolution.

12. Implications of horizontal gene transfer to risk assessment of transgenic micro-organisms

40. The occurrence of horizontal gene transfer among bacteria has important implications in the risk assessment of transgenic bacteria. The fact that horizontal gene transfer among bacteria is known to occur in the environment dictates the necessity for evaluation of the potential for subsequent gene transfer of introduced genetic sequences from a transgenic bacterium to indigenous micro-organisms when that bacterium is released into the environment. Transgenic bacteria released into the environment, like their unmodified parent organism, can also capture mobile genetic elements which might improve their adaptation to environmental stresses and thereby enhance their fitness. There are two important concepts in the risk evaluation of the potential for gene transfer from transgenic micro-organism. The first is an exposure component of risk that examines both the likelihood of transfer and the potential extent (*i.e.*, range of recipient organisms) of gene transfer from a transgenic micro-organism to other micro-organisms in the environment resulting from the unique design or construction of the transgenic micro-organism with consideration of the parental micro-organism, the introduced genes, the method(s) of insertion and the environmental habitat into which the GEM is released. The other necessary component in the risk assessment of transgenic micro-organisms in relation to gene transfer is the evaluation of any adverse effects should gene transfer occur. The evaluation of the potential consequences resulting from gene transfer is the more important consideration in an analysis of risks associated with gene transfer, and therefore, will be discussed first.

12.1. Potential adverse effects of horizontal gene transfer from a transgenic bacterium to indigenous micro-organisms

41. The risk associated with the horizontal gene transfer of transgenes from a transgenic bacterium is dependent on the likelihood of any adverse effects resulting from the acquisition of the transgenes in other

micro-organisms. If any phenotype associated with the inserted gene(s) is potentially unwanted in another host background, such as toxicity, pathogenicity, increased virulence, resistance to antibiotics, competitive advantage, utilisation of novel substrates, or greatly expanded host range, then a close examination of the potential for gene transfer is warranted. On the other hand, if the inserted gene(s) of interest does not impart any adverse effect or novel function, then evaluation of the exposure components of risk are less important. Likewise, if there is already a significant existing gene pool in the environment for genes imparting a particular inserted trait (*e.g.*, degradative pathways such as SAL, TOL, and NAH), then there may be little concern even if gene transfer from the transgenic bacterium to other micro-organisms in the environment readily occurs.

12.2. Likelihood and extent of gene transfer

12.2.1 Construction of the transgenic micro-organism: Implications for horizontal gene transfer

42. The method of construction of a transgenic bacterium may have an influence upon the likelihood of gene transfer from that bacterium to other micro-organisms in the environment. The potential for transfer by any of the bacterial mechanisms of horizontal gene transfer (*i.e.*, conjugation, transformation, and transduction) warrants consideration in this analysis.

43. *Genes introduced using plasmid vectors* - The use of plasmids as vectors that are retained as intact extrachromosomal elements in the transgenic bacterium may allow for likely horizontal gene transfer from that bacterium to other bacteria with the subsequent expression of acquired genes. Both the likelihood and the extent of transfer of a plasmid used as a vector to create a transgenic bacterium, as with plasmids in naturally-occurring bacteria, are dependent upon a number of factors of the plasmid itself, such as host range, the presence of resident plasmids in a potential recipient, and characteristics of potential environmental recipients as discussed previously in this document. Evaluations of the likelihood and extent of transfer of a plasmid vector from the transgenic micro-organism to indigenous micro-organisms through conjugation must take into account whether the plasmid in the final transgenic micro-organism construct still has an intact mobilisation (nick) site and transfer (*tra*) genes on the plasmid. Disruption of these genes may render a conjugative plasmid non-self-transmissible. However, consideration must also be given to the possibility for missing *tra* functions being provided *in trans* by another self-transmissible plasmid.

44. The use of plasmid vectors for insertion of genes into transgenic micro-organisms may also result in transfer of those genes by the other mechanisms of horizontal gene transfer such as transformation and by transduction. However, the frequency of plasmid transfer through these mechanisms is probably much less than that which would be expected by conjugation of self-transmissible plasmids. The size of the plasmid vector may influence its ability to be transferred by transformation, as there is less likelihood that larger plasmids would remain intact for extended periods in the environment where DNA is subject to degradation by nucleases. Likewise, there are physical limitations in the size of nucleic acid sequences that can be carried by transducing phages. Still, these mechanisms of horizontal gene transfer must be considered in the biosafety evaluation of transgenic micro-organisms.

45. *Genes inserted into the bacterial chromosome* - Gene transfer from transgenic micro-organism constructed by the stable incorporation of the inserted genes of interest into the recipient bacterial chromosome may not present as much of a concern as genes introduced on conjugative plasmid vectors. Chromosomal insertion has been successfully accomplished by a variety of different methods such as the use of suicide plasmids or conditional replicons, or through transposon activity. Chromosomal insertion of genes into a recipient may also be accomplished through other mechanisms such as transduction or transformation, or through artificial transformation techniques such as electroporation or use of a gene gun.

Regardless of the mechanism, the introduced DNA is integrated at low frequencies into the chromosome by homologous recombination or even through illegitimate/non-homologous recombination.

46. It should be noted, however, that even though chromosomal integration may decrease the frequency or likelihood of horizontal gene transfer from the transgenic bacterium to other micro-organisms, there is still the potential for gene transfer to occur. Some regions may be inherently more mobile, *e.g.*, genomic islands. Transposition could occur that may cause instability of the inserted gene from one location to another on the chromosome, or even to other replicons within a cell. The evolutionary importance of these processes is illustrated by the presence in microbial populations of genomic islands (including pathogenicity islands) that possess independent mobility through genes encoding integrases and transposons. These islands are examples of known vehicles for transfer of genes from chromosomal regions. They are generally regarded as mobilising components of the flexible gene pool, as opposed to "core genes" (Dobrindt *et al*., 2004) which often code for essential functions and appear to be less subject to mobilisation. In addition, transducing phages also can transfer chromosomal genes. Transformation of chromosomal pieces of DNA from dead cells may still occur. Although the frequency of transfer of chromosomal genes is expected to be lower than that for genes carried on self-transmissible plasmids, consideration of the mobile elements contained within the chromosome, which may lead to instability and thus transfer, must also be considered in risk assessment.

12.2.2 Other factors influencing horizontal gene transfer

47. There are a number of factors beyond the unique construction methods and final location of introduced genes within the genome of a transgenic bacterium that affect the likelihood and extent of horizontal gene transfer of inserted genes to indigenous micro-organisms. These include (1) the gene itself, including its phenotypic trait, especially if it confers a selective advantage to a recipient, (2) the inherent capability of the parental bacterium to transfer genes in general, (3) the ability of the transgenic bacterium to survive in the environment, (4) the presence of suitable recipient bacteria in the environment, and (5) various environmental factors that may affect microbial activity and transfer such as water content, nutrient status, clay mineralogy, pH, etc. The common experience of using transgenic micro-organisms in laboratory and field tests is a failure to maintain their presence or to transfer the introduced genes unless specifically enhanced for HGT.

48. As previously mentioned, the trait imparted by a gene(s) is important in assessing whether gene transfer may occur. Genes encoding traits conferring a selective advantage are likely to transfer and establish in bacterial communities regardless of genome location. The existence in microbial populations of genomic islands conferring traits such as pathogenicity, symbiosis, fitness, or resistance is evidence that such traits that are beneficial or useful to the host are commonly transferred (Dobrindt *et al*., 2004). However, novel genes that provide no competitive advantage to a new host may be lost, or not expressed. Some transgenic micro-organisms have been shown to have reduced fitness compared to their unmodified parental strains, which in some cases can be attributed to a metabolic drain on the cell. In other cases, some transgenic micro-organisms have exhibited decreased fitness due to cytotoxic effects of inserted genes. Since gene transfer is affected by the physiological state of the cell, the gene itself may be important in predicting gene transfer.

49. The inherent capability of the parental bacterium used in the creation of a transgenic micro-organism to exchange genes may influence the potential for horizontal gene transfer. Numerous bacterial species in the environment are known as natural gene exchangers. For example, pseudomonads are notorious for transfer of degradative genes (mostly contained on plasmids) among members in various genera. Closely related genera, such as *Pseudomonas* and *Burkholderia* can readily exchange, most likely due to the existence of the proper host machinery for both transfer and expression of newly acquired genes.

Broad host range plasmids such as IncP-1 plasmids (Pukall *et al*., 1996, Top and Springael, 2003) but also the newly discovered groups of BHR such as pIPO2 (Van Elsas *et al*., 1998; Tauch *et al*., 2002) transfer readily between distantly related bacteria. It is important to recognise the plasticity of the bacterial genomes.

50. An accurate taxonomic identification of the recipient micro-organism used in creation of a transgenic micro-organism may assist in assessing the potential for gene transfer from the transgenic micro-organism to other micro-organisms in the environment. The importance of utilising the proper phenotypic and genotypic tests and methods for accurate identification of bacteria has been previously addressed (OECD, 2003).

51. For several mechanisms of horizontal gene transfer to occur, the transgenic bacterium must survive, at least transiently. If the transgenic micro-organism is unlikely to survive in the environment, the potential for transfer is greatly diminished, at least for the mechanisms of conjugation and transduction. There is still the possibility for transformation of DNA released from dead cells, although without multiplication of the bacteria, cells would be limited in number. As previously discussed, the persistence of naked DNA in the environment is dependent on a number of factors including adsorption to bacterial membranes, clay minerals, and other surfaces which provide protection from degradation by nucleases.

52. If the transgenic bacterium survives well in the environment, then the presence and concentration of appropriate recipients affects the extent of potential gene transfer. As previously discussed in this document, there are "hot spots" in soil and water environments and on plant surfaces where microbial concentrations are increased, and thus, there is greater potential for horizontal gene transfer to occur, especially through conjugation. Conjugation could also be stimulated at higher cell densities by quorum sensing systems, *e.g.*, in *Agrobacterium* (Miller and Bassler, 2001). Likewise, horizontal gene transfer by transduction and transformation is more likely to occur in areas of greater concentrations of micro-organisms.

53. Environmental factors influence the potential for horizontal gene transfer from a transgenic micro-organism to other micro-organisms in the environment. The physiological state of both the donor and recipient micro-organisms may be influenced by a number of abiotic factors, such as water content, temperature, nutrient status, and soil pH. In addition, environmental factors may affect the development of competence for transformation, and abiotic variables such as clay type and content or organic matter content may provide protection of free nucleic acid sequences from degradation. *In situ* acquisition of mobile genetic elements that might confer selective traits by the transgenic micro-organism has recently been shown for the transgenic *Pseudomonas fluorescens* SBW25EeZY6KX under field conditions in two consecutive years (Lilley and Bailey, 1997a). Transconjugants which acquired Hg^r plasmids were isolated from the rhizosphere and the phyllosphere only at a certain time of plant development. The acquisition of new genetic information might change the characteristics of a transgenic micro-organism. The type and frequency of post-release acquisition of additional genetic information will depend on the environmental habitat to which the transgenic micro-organism is released but also on the host organism.

12.2.3 Expression of acquired genes

54. Horizontal transfer of genes is no guarantee that a particular gene, or all transferred genes, will be efficiently expressed in a new host organism. Transcription is required for expression, and therefore, the new host must possess an RNA polymerase that will recognise the promoter and initiate RNA synthesis. This is not always the case, and the efficiency may vary in different recipient micro-organisms. In addition, a recipient micro-organism may or may not contain the appropriate enzymes for post-translational modification of the protein to produce a biologically active molecule. It should be noted, however, that

even if the new host micro-organism does not possess the required enzymes for expression of the foreign gene, the gene sequence still may reside in the genome, and thus may subsequently be transferred to other micro-organisms in the environment capable of efficiently expressing the gene. However, it is often the case that genes that do not confer an advantage to the recipient micro-organism may be lost from the genome, and therefore, subsequent transfer may not be of concern.

12.3. Conclusions

55. The risk assessment of a transgenic bacterium must consider the potential for transfer of introduced genes to other micro-organisms in the environment. Of greater importance, however, is consideration of whether there would be any adverse consequences posed if gene transfer from a transgenic bacterium occurred. If no adverse effects can be envisioned, then prediction of the likelihood, frequency, and extent of gene transfer are of less concern. If detrimental consequences were to occur if genes were transferred to indigenous micro-organisms, then the exposure components of risk must be carefully evaluated, both from a theoretical perspective depending on potential mechanisms of transfer, and from a real-life perspective given natural barriers to gene transfer in the environment.

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