Chapter 7
Horizontal Gene Transfer Between Bacteria Under Natural Conditions

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Abstract Conjugative plasmid transfer is the most important mechanism for bacteria to deliver and acquire genetic information to cope with rapidly changing environmental conditions. An update of knowledge of conjugative plasmid transfer in aquatic and terrestrial habitats, including environments of particular concern such as agricultural areas and contaminated soils and sediments, is presented. Environmental factors affecting horizontal gene transfer in nature are discussed. Recent advances in the design of in situ monitoring tools to assess conjugative plasmid transfer in nature and laboratory model systems to simulate environmental conditions are critically reviewed. The impacts of horizontal gene transfer on biodegradation as well as recent approaches to model conjugative plasmid transfer in complex microbial communities are presented.

7.1 Introduction

The "horizontal gene pool" refers to genetic information accessible to more than a single bacterial species, potentially resulting in phenotypes of one being acquired by another. This pool includes genes of mobile genetic elements (MGEs) and genes that are not mobile themselves, but may be mobilized by MGEs (Slater et al. 2008). Plasmids, bacteriophages, conjugative transposons, and integrative conjugative elements (ICE) are examples of MGEs. Plasmids as self-replicating MGEs generally provide accessory, but not essential functions to their hosts. In particular, traits that confer adaptations to locally restrictive conditions tend to be clustered on plasmids.

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In spite of the energetic burden imposed upon the host cell metabolism, plasmids can be considered as desirable elements for their host providing a mechanism for initiation of functions that are required for survival under environmental stress conditions but are dispensable in the absence of stress effectors (van Elsas et al. 2000).

Bacterial conjugation is one of the most important means of gene delivery enabling adaptation of bacteria to changing environmental conditions, including spread of antibiotic resistance genes, thereby generating multiple antibiotic-resistant bacteria.

As microorganisms occupy and adapt themselves to different ecological niches within the biosphere, their activities control global homoeostasis in large part. Through its effects on microbial adaptation, horizontal gene transfer (HGT) poses both challenges and opportunities for the control of global human and environmental health (Smets and Barkay 2005). The mobile gene pool or "mobilome" spans all kingdoms of life.

This chapter focuses on the bacterial mobilome, the gene pool available for transfer from one bacterium to the other and for acquisition by bacteria from other organisms in the same environment. The chapter is divided into sections dealing with HGT in different natural and anthropogenic habitats including soil, sediments, and aquatic environments Research performed by several groups on HGT modeling is summarized. Promising approaches to monitor HGT without cultivation of the cells as well as valuable approaches to assess HGT frequencies under natural conditions or conditions mimicking nature are discussed. The chapter ends with a description of technological prospects provided by transmissible traits encoded on MGE with respect to biodegradation and bioremediation in contaminated habitats, and perspectives for HGT research.

7.2 Horizontal Gene Transfer in Soil, Sediments, and Other Solid Surfaces

An excellent summary of the ecology of plasmid transfer was published by van Elsas et al. (2000). Van Elsas and coworkers issued key questions to be answered in the field of ecology of HGT. One of the most important questions is how efficiently do plasmids spread in the environment and how is this spread affected by environmental factors.

Van Elsas et al. (2000) defined a series of key abiotic and biotic factors that affect plasmid host fate in natural environments, presumably having a net effect on HGT. The stimulating abiotic factors include, among others, presence of nutrients, presence of colonizable surfaces, soil texture (e.g., high clay content favors HGT due to protection of plasmid hosts), physiological temperatures, presence of oxygen for aerobic microorganisms, etc. Biotic factors that enhance HGT include plant roots and other nutrient-rich colonizable surfaces, as well as soil animals

offering colonizable surfaces and interior environments, such as the gut of soil insects where plasmid transfer was demonstrated (Hoffmann et al. 1998, 1999). Many studies have reported the occurrence of conjugative transfer (mediated by plasmids or conjugative transposons) between bacteria in soil (e.g., Krasovsky and Stotzky 1987; Schofield et al. 1987; van Elsas et al. 1988a, b; Richaume et al. 1989; Henschke and Schmidt 1990; Smit et al. 1991, 1993; Pukall et al. 1996; Götz and Smalla 1996; van Elsas et al. 1998; van Elsas and Bailey 2002; Smalla et al. 2006; Ansari et al. 2008; Malik et al. 2008; Sobecky and Coombs 2009). These studies have conferred important knowledge on HGT in natural habitats; however, all possess natural restrictions or limitations. They focused on a specific habitat and/or a particular MGE or a particular class of MGEs, in most cases conjugative plasmids.

Van Elsas and Bailey (2002) reviewed the impact of different experimental approaches and the influence of key environmental factors on HGT in soil and the phytosphere. They demonstrated how structured bacterial communities such as biofilms and selective pressure affect HGT frequencies in natural bacterial consortia.

7.2.1 Environmental Factors Affecting HGT in Nature

Microbial growth in most natural environments is restricted by the quantity of nutrients present, which can dramatically limit population densities and activity (van Elsas and Bailey 2002). In soil, in particular, plant surfaces were shown to provide conditions for microbial colonization, mixing, and bacterial activity, resulting in locally enhanced densities of bacterial cells. These sites offer favorable conditions for gene exchange and have been named "hot spots" for HGT (van Elsas et al. 2000). Hot spots for HGT processes in soil include rhizosphere and belowground plant tissue (e.g., Pukall et al. 1996; Lilley and Bailey 1997; Kroer et al. 1998; van Elsas et al. 2000; van Elsas and Bailey 2002), the phyllosphere (e.g., Björklöf et al. 1995; van Elsas et al. 2000; Kay et al. 2002), manured soil (e.g., Götz and Smalla 1996; Heuer and Smalla 2007; Heuer et al. 2009) as well as guts of soil animals such as *Collembola* and earthworms (Daane et al. 1996; Hoffmann et al. 1998; Thimm et al. 2001).

7.2.2 Tools to Study Horizontal Gene Transfer in the Environment

Three different types of tools are applied to study HGT in nature:

1. *Direct disruptive tools*. These methods include extraction of bacteria from the environment and cultivation of the bacteria on selective media, followed by

molecular analysis. They have been applied to microcosms simulating plasmid transfer conditions in soil and the rhizosphere (e.g., Hoffmann et al. 1998; Kroer et al. 1998; Lilley et al. 2003) and to studies in the field, e.g., to investigate plasmid transfer in the phytosphere (e.g., Lilley et al. 1994; Lilley and Bailey 1997; van Elsas et al. 1998).

2. *Indirect tools*. These methods include plasmid DNA isolation, PCR on genes encoded by MGEs, such as antibiotic resistance genes and key transfer factors, and sequence analysis of MGEs or parts thereof. They have been applied to different terrestrial environments to detect sequences of MGEs supporting evidence of gene transfer potential. The indirect tools do not provide evidence of plasmid transfer, but they provide evidence of the presence of conjugative plasmids and of the respective transfer genes. Molecular detection of transfer genes and plasmid DNA isolation does not prove that gene transfer takes place or has taken place in the respective habitats. However, it supports evidence of gene transfer potential (e.g., Götz and Smalla 1996; Levin and Bergstrom 2000; Ochman et al. 2000; Smalla et al. 2000; Mendum et al. 2001; Ansari et al. 2008; Malik et al. 2008; Ansari 2009).
3. *Direct, nondisruptive tools*. These methods include fluorescence monitoring tools such as the use of plasmid donors with repressed *gfp*, coding for the Green Fluorescent Protein (e.g., Sørensen et al. 2003). These tools have been applied in particular to detect plasmid transfer events in biofilms (e.g., Christensen et al. 1996, 1998; Heydorn et al. 2000). Recent developments on nondestructive techniques to quantify plasmid transfer are summarized in the section "Monitoring HGT and assessing transfer frequencies".

7.3 Plasmid-Mediated Gene Mobilization in Soil

Plasmids drive HGT in soil; however, information on the diversity of plasmids and other MGEs in soil and the phytosphere is still scarce. Depending on plasmid isolation protocol, plasmids with different characteristics with respect to Inc group, host range, antibiotic and heavy-metal resistance, and conjugative and mobilizable abilities can be obtained. The most effective methods to obtain conjugative plasmids with plasmid mobilization capacity are bi- and triparental exogenous isolation. Conjugative plasmids are captured directly from environmental samples into recipient strains grown under selective laboratory conditions (Bale et al. 1988b; van Elsas and Bailey 2002). These tools have been successfully applied to soil and phytosphere habitats (e.g., Lilley et al. 1994; van Elsas et al. 1998; Malik et al. 2008). pIPO2 was shown to self-transfer and mobilize IncQ plasmids to various Gram-negative bacteria in the wheat rhizosphere under field conditions (van Elsas et al. 1998). Mercury-resistance plasmids that were able to mobilize IncQ plasmids such as RSF1010 were also found (van Elsas et al. 2000). Prevalence of these plasmids seemed to be enhanced under conditions of mercury stress.

7.3.1 Horizontal Gene Transfer in Metal- and Radionuclide-Contaminated Soils and Sediments

The presence of conjugative plasmids and antibiotic-resistance genes in anthropogenic soils from India and Germany by antibiotic resistance gene and key transfer factor-specific PCR and Southern hybridization was investigated in our laboratory. The abundance of resistance factors and broad-host-range conjugative plasmids in an urban park and an abandoned sewage field in Germany were compared with those from four different Indian sites, three agricultural fields with distinct irrigation history (irrigation with industrial wastewater from tannery or steel industries), and one agricultural field irrigated with groundwater (Malik et al. 2008). Samples from the abandoned sewage field and all the Indian soils demonstrated the occurrence of IncP-specific plasmid sequences like $oriT_{\text{IncP}}$ and the replication gene *trfA*, whereas in soil samples from the urban park, no IncP sequences were detected. Biparental exogenous plasmid isolation with bacteria detached from contaminated soils showed prevalence of conjugative IncPβ plasmids in the strongly polluted German site (abandoned sewage field) and the Indian agricultural field which had received wastewater from steel industries for many years. A similar conclusion was obtained by studies on multiple antibiotic- and heavy-metal-resistant bacterial isolates from highly heavy-metal-contaminated Indian soils for the presence of conjugative plasmids from Gram-negative bacteria. The presence of conjugative/mobilizable IncP plasmids in the isolates indicated their gene-mobilizing capacity with implications for potential dissemination of introduced recombinant DNA (Ansari et al. 2008).

Smalla et al. (2006) detected increased abundance of IncP-1β plasmids and mercury-resistance genes in mercury-polluted river sediments. They investigated river sediment samples from two mercury-polluted and two nonpolluted or less-polluted areas of a river in Kazakhstan for the presence of mercury-resistance genes and broad-host-range plasmids by PCR. An increase of the degree of mercury pollution corresponded to an increased abundance of mercury-resistance genes and of IncP-1β replicon-specific sequences detected in total community DNA (Smalla et al. 2006). Three different IncP-1β plasmids (pTP6, pTP7, and pTP8) were captured from contaminated sediment by the triparental exogenous plasmid isolation method. The plasmids conferred mercury resistance to their host, and the presence of a mercury-resistance transposon on these plasmids was demonstrated by hybridization. The nucleotide sequence of pTP6 revealed a backbone almost identical to that of the classical IncP-1β plasmid R751 (Smalla et al. 2006). This study provided further evidence of the role of IncP-1β plasmids in mediating maintenance and spread of adaptive traits such as mercury resistance, in bacterial communities.

Sobecky and Coombs (2009) summarized the state of the art of HGT in metal- and radionuclide-contaminated soils. Metal and radionuclide contamination in soils and in the subsurface poses a serious challenge to bacterial growth and survival because these contaminants cannot be transformed or biodegraded into nontoxic forms as it often occurs with organic xenobiotics (Sobecky and Coombs 2009). HGT

has played a major role in the dissemination of metal-resistance determinants among microbial communities (Sobecky and Coombs 2009). Metal-resistance genes were first detected on plasmids from diverse bacteria (Summers and Silver 1972; Silver et al. 1981). Subsequently, it was shown that transposons promote the acquisition of these plasmid-encoded metal-resistance genes (Liebert et al. 1999). Mercury-resistance operons often encode concomitant antibiotic resistance genes (Liebert et al. 1999; Gilmour et al. 2004). Baker-Austin et al. (2006) and Wright et al. (2006) demonstrated that heavy-metal contamination due to anthropogenic sources contributes to the dissemination of antibiotic-resistance genes by either coselecting for antibiotic-resistant bacteria carrying metal-resistance genes, located on the same MGE, or by selecting for cross-resistance encoded by multidrug efflux pumps exporting metals and antibiotics (Baker-Austin et al. 2006; Wright et al. 2006).

One of the best characterized metal-resistance loci is the *mer* operon, consisting of up to seven genes required for transport, catalysis, and regulation of mercury resistance (Barkay et al. 2003). Exogenous plasmid isolation was applied to isolate conjugative mercury-resistance plasmids from bacterial soil populations (Sobecky and Coombs 2009). Five different novel Hg^R plasmid groups were detected in the rhizosphere and phyllosphere of sugar beets (Lilley et al. 1996). A study on the soil bacterial populations associated with wheat roots showed that in soils amended with mercury, novel plasmid groups conferring Hg^R were recovered by exogenous plasmid isolation (Smit et al. 1998). This phenomenon emphasizes the endemic nature of MGEs conferring Hg^R resistance among soil microbial communities.

Three mechanisms that promote microbial heavy-metal resistance or tolerance are known: (1) metal reduction, (2) metal complexation, and (3) ATP-dependent metal efflux (Sobecky and Coombs 2009). The P_{IB}-type of prokaryotic heavy-metal-translocating ATPases detoxifies the bacteria by exporting Cd(II), Co(II), Pb(II), Ni(II), and Zn(II) (Sobecky and Coombs 2009). P_{IB}-type ATPase genes have been detected on MGEs from Gram-positive (Nucifora et al. 1989; O'Sullivan et al. 2001) and Gram-negative bacteria (Mergeay et al. 2003). Dissemination of horizontally acquired P_{IB}-type ATPase genes was shown by Sobecky and Coombs (2009).

Arsenic (As) occurs in four different oxidation states, As^{+5}, As^{+3}, As^{0}, and As^{-3}. It is a micronutrient used by a variety of microorganisms for cell growth and metabolism (Sobecky and Coombs 2009). Prokaryotic metabolic activity has been shown to be important in the transformations and subsequent mobilization/immobilization of As compounds (Stolz et al. 2006). The *ars* operon encodes a detoxification pathway for As, which can be chromosomally or plasmid-encoded (Sobecky and Coombs 2009). The operon contains numerous genes including *arsC* encoding arsenate reductase, which reduces arsenate to arsenite (Stolz et al. 2006). Phylogenetic analysis of more than 400 *arsC* sequences supported the role of HGT in the evolution and dissemination of arsenate reductase (Jackson and Dugas 2003).

Radionuclides cause severe environmental contamination problems for several reasons: (1) many radionuclides are heavy metals, and exposure to cells results in toxicity effects in addition to damage caused by radioactive decay; (2) radionuclides cannot be broken down or detoxified by transformation; (3) radionuclides are often present together with other environmental contaminants. This means that any

surviving organism in affected environments must be multiple-contaminant resistant; and (4) bacteria have only limited resistance mechanisms for radionuclides (Sobecky and Coombs 2009).

Dissimilatory metal-reducing bacteria such as *Geobacter sulfurreducens* (e.g., Lovley et al. 1991; Lloyd et al. 2000) and sulfate-reducing bacteria such as *Desulfovibrio desulfuricans* (Lloyd et al. 1999) contain electron shuttle systems that immobilize radionuclides by reduction to their less mobile forms.

Reduction of radionuclides and heavy metals such as Cr(VI) is carried out by two possible mechanisms. Indirect reduction could take place when Fe(II), Mn(II), and H_2S are generated by microbes during anaerobic respiration. The oxidation of these compounds to Fe(III), Mn(IV), and SO_4^{-2} could work to reduce metals such as U(VI) and Tc(VII). Indirect reduction has not yet been shown in situ, however. The alternative mechanism is direct enzymatic reduction, a process not fully understood (Sobecky and Coombs 2009). However, it is known that c-type chromosomes play an important role in dissimilatory metal-reducing bacteria (e.g., Shelobolina et al. 2007; Marshall et al. 2008) and in sulfate-reducing bacteria (Lovley et al. 1993; Payne et al. 2004). There is no direct evidence of HGT of genes required for enzymatic reduction; however, studies with c-type cytochromes demonstrated that HGT of these cytochromes can occur (Bertini et al. 2007; Sobecky and Coombs 2009). Analysis of 235 bacterial genomes revealed c-type cytochromes in nine cyanobacteria, *G. sulfurreducens*, and *Nitrosomonas europaea* (Sobecky and Coombs 2009).

7.3.2 Horizontal Gene Transfer in Mixed Waste Sites

Mixed waste in this section refers to anthropogenic contamination consisting of organic chemicals and radionuclides. Mixing of more than one contaminant at a waste site is important, as cocontaminants may interact with each other to enable or interfere with chemical transformation or contaminant transport. Variation in electron acceptors can result in the generation of different redox zones over small spatial scales (Barber et al. 1992; Cozzarelli and Weiss 2007; Sobecky and Coombs 2009).

Metal resistance and catabolic genes are often encoded on MGEs (for reviews see Liebert et al. 1999; Mergeay et al. 2003; Springael and Top 2004). Transposons that have been sequenced from environmental samples appear to encode only catabolic genes (for a review see Wyndham et al. 1994) or metal-resistance genes (e.g., Mindlin et al. 2001; Kholodii et al. 2002). A small number of plasmids contain genes for both (Sobecky and Coombs 2009). Of these, pJP4, pWW0, and pUO1 are self-transmissible plasmids (Kawasaki et al. 1981; Neilson et al. 1994; Pinedo and Smets 2005). Mobilization of pJP4 was demonstrated in soil (Neilson et al. 1994) and in bioreactors containing 2,4-D or 2,4-D and cadmium (Newby et al. 2000; Sobecky and Coombs 2009). It appears that exposure to toxic compounds such as 2,4-D and cadmium does not have a stimulating effect on the conjugative transfer of large catabolic plasmids (Sobecky and Coombs 2009).

Several cases of HGT among high and low G/C Gram-positive bacteria from mixed-waste have been reported. Most bacteria harbored large plasmids and could also tolerate toxic concentrations of U(IV) at low pH (Martinez et al. 2006). The frequency of HGT was higher among isolates from the contaminated site than from an uncontaminated site (Coombs and Barkay 2004).

7.3.3 Horizontal Gene Transfer in Agricultural Soils

Genomic approaches have revealed a large diversity of MGEs in soil and plant-associated bacteria, including plasmids, prophages, pathogenicity islands, and integrons. Pathogenicity islands are MGEs that account for rapid changes in virulence potential. They are known to have contributed to genome evolution by HGT in many bacterial pathogens (Dobrindt et al. 2004). Integrons are assembly platforms – DNA elements that acquire open reading frames embedded in exogenous gene cassettes – and convert them to functional genes by ensuring their correct expression (Mazel 2006; Heuer and Smalla 2007). Approximately 18% of bacterial isolates from the phytosphere of sugar beets were shown to harbor plasmids (Powell et al. 1993). Many were able to mobilize nonself transmissible IncQ plasmids (Kobayashi and Bailey 1994).

The exogenous isolation of MGEs was applied to capture MGEs from soil and phytosphere microbial communities (Smalla and Sobecky 2002). Antibiotic resistance or mercury resistance was often used as selective markers to exogenously isolate conjugative plasmids from the phytosphere of different crops (e.g., Lilley et al. 1996; Lilley and Bailey 1997; Smit et al. 1998; Schneiker et al. 2001; Malik et al. 2008) and from mercury-polluted soils (Dronen et al. 1998) in Gram-negative plasmid recipients. Biodegradative genes encoded on MGEs were captured from soils treated with 2,4-D, but not from untreated controls (Top et al. 1995, 1996). Two different cultivation-independent approaches were used to isolate naphthalene-catabolic genes from oil-contaminated soil in Japan (Ono et al. 2007). One approach was the construction of a broad-host-range cosmid metagenomic library; the other involved exogenous plasmid isolation. A cosmid clone was obtained that carried a naphthalene-catabolic pathway operon for conversion of naphthalene to salicylate. The operon was similar to the corresponding operon on the IncP-9 naphthalene-catabolic plasmid pDTG1. Using the exogenous approach the microbial soil community was mated with a *Pseudomonas putida* recipient. Transconjugants had acquired either a 200- or 80-kb plasmid containing all the naphthalene-catabolic genes for complete degradation of naphthalene. Both plasmids belong to the IncP-9 incompatibility group, and the naphthalene-catabolic genes are highly similar to those of other IncP-9 plasmids, namely, pDTG1 and pSLX928-6 (Ono et al. 2007).

Miyazaki and coworkers determined the nucleotide sequence of the exogenously isolated plasmid pLB1 involved in γ-hexachlorocyclohexane degradation (Miyazaki et al. 2006). pLB1 was isolated from hexachlorocyclohexane-contaminated soil and

transferred from *Sphingobium japonicum* to other alpha-proteobacterial strains by conjugative transfer. Thus, pLB1 may contribute to the dissemination of genes for γ-hexachlorocyclohexane degradation in agricultural soils (Miyazaki et al. 2006).

Conjugative plasmids encoding multiple antibiotic resistance were captured from animal manure used for soil fertilization (Smalla et al. 2000; Heuer et al. 2002, 2008; van Overbeck et al. 2002; Heuer and Smalla 2007; Binh et al. 2007, 2008). van Elsas et al. (1998) isolated mobilizing plasmids from the rhizosphere of wheat plants by using microbial communities detached from the rhizosphere as donors in triparental matings. Plasmid pIPO2 was isolated in *Ralstonia eutropha* on the basis of its mobilizing capacity. Replicon typing and plasmid sequencing showed that this 45-kb cryptic plasmid was not related to any of the known broad-host-range plasmids except plasmid pSB102 (Schneiker et al. 2001). Sequencing of plant-associated bacteria has revealed that many phytopathogenic and symbiotic bacteria harbor plasmids (Vivian et al. 2001; Zhao et al. 2005; Sundin 2007; Crossman et al. 2008; Li et al. 2008; Ding and Hynes 2009), pathogenicity or symbiosis islands (Arnold et al. 2003; Ramsay et al. 2006; Büttner et al. 2007; Nandasena et al. 2007; Nakatsukasa et al. 2008), or integrons (Szczepanowski et al. 2004; Gillings et al. 2005).

Agersø et al. (2006) investigated the effect of tetracycline residues in pig manure slurry on tetracycline-resistant bacteria and the tetracycline resistance gene *tet*(M) in soil microcosms. Four different types of microcosms were established, supplemented with combinations of pig manure slurry and a tetracycline-resistant *Enterococcus faecalis* strain encoding the *tetM* resistance gene. The concentration of both tetracycline-resistant bacteria (total CFU) and tetracycline-resistant enterococci declined rapidly in all four types of microcosms. *tet*(M) was detected longer than tetracycline-resistant enterococci could be isolated. This result could be due to the presence of viable but not culturable (VBNC) bacteria encoding *tet*(M), HGT of *tet*(M) to indigenous soil bacteria, or presence of free DNA, e.g., attached to soil particles. The concentration of tetracycline was approximately stable throughout the study, but the antibiotic concentration had no effect on prevalence of tetracycline-resistant bacteria (Agersø et al. 2006). The tetracycline residues present in the microcosms originated from pig manure slurry resulting from therapeutic treatment of the pigs. Tetracycline concentrations were similar to the actual concentration in manured agricultural soil. At this concentration, tetracycline did not appear to select for tetracycline-resistant bacteria, but it is degraded slowly in soil and may accumulate over time if manure containing tetracyclines is regularly amended to the soil (Agersø et al. 2006). As *tet*(M) was detected much longer than the original *E. faecalis* host, the resistance genes might form an antibiotic resistance reservoir in soil (Agersø et al. 2006).

Toomey et al. (2009) studied the HGT of antibiotic-resistance genes (plasmid- and transposon-encoded) between wild-type dairy isolates of lactic acid bacteria using an alfalfa sprout model. The plant model provided an environment that appeared to promote high transfer frequencies between all lactic acid bacteria pairs tested. Transfer frequencies ranged from 4.7×10^{-4} to 3.9×10^{-1} transconjugants per recipient. Dairy cultures can act as a source of MGEs encoding antibiotic resistance that can

be spread with high frequency to other lactic acid bacteria in plant environments (Toomey et al. 2009).

7.4 Horizontal Gene Transfer in Aquatic Environments

As for soil and other natural environments, the frequency of conjugative plasmid transfer among bacteria in aquatic environments appears to be controlled by the characteristics of hot spots (van Elsas et al. 2000). Aquatic ecosystems can be divided into different habitats, (1) the free (bulk) water phase, (2) the colonizable suspended matter, (3) sediment or sewage, (4) stones and other surfaces carrying biofilms (termed the epilithon) (Hill et al. 1996), and (5) aquatic animals. Availability of nutrients as well as colonizable surfaces is important due to support of large densities of metabolically active bacteria (Hill et al. 1994; Muela et al. 1994; van Elsas et al. 2000). Suspended matter is a preferred site for bacterial growth, resulting in bacterial densities higher than that in bulk water. Sediments rich in organic material can support bacterial population densities approximately three orders of magnitude higher than those found in bulk water (Ashelford et al. 1997; van Elsas et al. 2000). Bacterial biofilm communities are found in the epilithon on stones in rivers or lakes (Lock et al. 1984) and in percolating filter beds, which are nutrient-rich environments that support high population densities of metabolically active bacteria (Gray 1992; van Elsas et al. 2000). Hence, plasmid transfer frequencies in aquatic environments seem to depend mainly on the possibilities for the formation of mixed donor–recipient colonies or biofilms (van Elsas et al. 2000). In natural environments, competing, grazing, or antagonistic microflora can impart a significant effect on HGT rates, as donor and recipient cell numbers and physiological activities can be severely affected. Bale et al. (1987, 1988a, b) and Hill et al. (1994) investigated the transfer of epilithon-derived plasmids between different *Pseudomonas* isolates. Due to antagonistic effects, transfer frequencies on sterile stones in broth were higher than those on epilithon-covered stones in river water.

7.4.1 Evidence of Plasmid Transfer in Aquatic Environments

Most HGT studies in aquatic environments have been performed in microcosms, as they provide the advantage of controllable study conditions (van Elsas et al. 2000). HGT frequencies in microcosms are often revealed to be consistent with those obtained in situ (Ashelford et al. 1995, 1997). Microcosms such as flasks, sediment columns, activated sludge units, sewage filter beds, or small chemostats are constituted of enclosed samples of the environment they mimic or of synthetic approximations of environmental samples. Indigenous microorganisms and other factors that provide complexity to the system, in particular colonizable surfaces and/or nutrient sources, may be present, and temperatures may be controlled or

manipulated. Although these microcosms are still different from the environment they represent, they are valuable, as they are simple, reproducible, flexible, and offer the possibility to control and/or adjust individual parameters (van Elsas et al. 2000). In microcosms and in some in situ experiments, plasmid transfer between different bacteria was demonstrated in drinking water (Sandt and Herson 1991), river water and epilithon (e.g., Bale et al. 1987; Hill et al. 1994; Muela et al. 1994; Shintani et al. 2008), lake water (O'Morchoe et al. 1988; Jones et al. 1991; Popova et al. 2005), seawater (e.g., Goodman et al. 1993; Barkay et al. 1995; Dahlberg et al. 1998; Sobecky and Hazen 2009), marine sediment (e.g., Breittmayer and Gauthier 1990; Sandaa 1993; Rasmussen and Sørensen 1998), and sewage and wastewater (e.g., Gealt et al. 1985; Lebaron et al. 1994; Ohlsen et al. 2003). Thus, plasmid transfer seems to be part of the natural lifestyle of bacterial cells inhabiting these environments (van Elsas et al. 2000).

7.4.2 *Evidence of Plasmid Transfer in Sewage Filter Beds and Activated Sludge Units*

Sewage filter beds and activated sludge units represent aquatic environments of extreme nutrient availability, microbial mixing, and competition. Therefore, plasmid transfer should occur at maximum rates in these systems. Sophisticated microcosms have been designed to mimic natural conditions of sewage filter beds and activated sludge units (van Elsas et al. 2000). Plasmid transfer in percolating filter beds was first studied by Ashelford et al. (1995). Plasmid transfer between different *P. putida* strains in the filter biofilm was observed (Ashelford et al. 1995, 1997).

Plasmid transfer studies were also performed in laboratory-scale activated sludge units by Mancini et al. (1987) and McClure et al. (1989). Mancini and coworkers studied conjugative plasmid transfer between laboratory *E. coli* K12 strains and between wastewater-isolated *E. coli* strains. Transconjugants were detected throughout the microcosm, with highest frequencies, 2.5×10^{-3} transconjugants per donor for laboratory strains, in the settled sludge (van Elsas et al. 2000). McClure et al. (1989) investigated the fate of a *P. putida* strain harboring the mobilizable plasmid pD10 in activated sludge units. They demonstrated pD10 mobilization to indigenous sludge bacteria. This demonstrates that mobilization of nonconjugative plasmids can readily occur in nutrient-rich environments.

7.5 Modeling of Conjugative Plasmid Transfer

Several attempts to model conjugative plasmid transfer and conjugative mobilization using different mathematical models with distinct simplifications on the complex process of plasmid exchange between two organisms have been reported. Some recent models are described briefly. Gregory et al. (2008a) used COSMIC-rules,

an individual-based model for bacterial adaptation and evolution to study virtual transmission of plasmids within bacterial populations. Their simulations showed the spread of resistance (R) plasmids, compatible and incompatible, by conjugative transfer. Three case studies were examined: transfer of an R plasmid within an antibiotic-susceptible population, transfer of two incompatible R plasmids, and transfer of two compatible R plasmids. Rules for plasmid transfer, e.g., cost rules for plasmid maintenance versus benefit rules for plasmid maintenance were set up (Gregory et al. 2008a). Simulations were carried out for all the three case studies. Transfer of R plasmids was demonstrated to occur in the simulations of all the three case studies. The results support the original concept of the authors (Gregory et al. 2008b), e.g., incompatibility could be predicted by this model to be an important limiting factor for plasmid spread in bacterial populations.

The ability to simulate plasmid transfer has applications in studies of adaptive evolution, dissemination of antibiotic-resistant bacteria (DeNap et al. 2004), and the ability of microbial populations to degrade xenobiotics (Basta et al. 2004).

Inoue et al. (2009) investigated the occurrence and persistence of transconjugants that have acquired self-transmissible plasmids via conjugation by a simulation model on conjugative plasmid transfer in soil. Two conjugative plasmids with broad-host-range in Gram-negative bacteria, RP4 and pJP4, were applied to transfer studies in soil microcosms. The simulation model incorporated the survival dynamics of the donors, recipients, and transconjugants, and the conjugative plasmid transfer dynamics. Bacterial survival was modeled as a simple growth/decay process. Bacterial conjugation was described based on the mass action model (Levin et al. 1979). Transconjugants were assumed to show similar survival characteristics as recipients with positive/negative effects resulting from plasmid acquisition. They were assumed to act as secondary plasmid donors with transfer rates differing from those of the original donors (Inoue et al. 2009). The microcosm experiments demonstrated that transconjugants occurred in soil even if the concentration of the original plasmid donors declined rapidly. The introduced plasmid can persist in the microbial community if the indigenous transconjugants are excellent plasmid donors with a broad spectrum of plasmid hosts (De Gelder et al. 2005) and/or high transfer frequency (Newby et al. 2000). The study of Inoue et al. (2009) was the first to model bacterial conjugation in complex microbial populations.

Sudarshana and Knudsen (2006) attempted to model plasmid mobilization between *E. coli* donors and *Pseudomonas fluorescens* recipients on pea seeds and roots. They developed a mathematical model to predict mobilization rates and to estimate the proportion of triparental matings in which plasmid mobilization occurs. The simple mathematical model was based on the mass action model of Levin et al. (1979) that was also applied by Inoue et al. (2009). The model assumes that matings occur among uniformly distributed donor and recipient cells and cells grow at the same constant rate. Although these assumptions are not fully met in heterogeneous ecosystems such as soil, modifications of the mass action model have been successfully applied to predict bacterial conjugation in soil, the rhizosphere, and the phyllosphere (Knudsen et al. 1988; Richaume et al. 1989; Clewlow et al. 1990).

The study of Massoudieh and coworkers focused on exploratory modeling of HGT among surface-associated *E. coli* in the subsurface (Massoudieh et al. 2010). They developed a model and experimental system to quantify HGT in biofilms formed on granular porous media in microflow chambers. Important characteristics of this sophisticated model are mentioned briefly. To track the kinetics of the partners in the conjugation process, four main states of the bacteria in the conjugative plasmid transfer process were considered: (1) donors, (2) recipients, (3) transconjugants, and (4) donors in the exhausted state (donors which have to recover before reinitiating conjugative transfer). The simulations confirmed the strong dependence of the transfer rate on the concentration of donors and recipients and considered attachment and detachment rates of the bacteria involved. Studies on sandy media and on glass beads will verify the model and quantify the characteristics of each of the processes considered in the model (Massoudieh et al. 2010).

7.6 Monitoring Horizontal Gene Transfer and Assessing Transfer Frequencies

Several excellent papers have been published on visualization of HGT between bacteria in vitro. Most of them apply fluorescent tools; e.g., Babic et al. (2008) presented an elegant study on direct visualization of HGT between single cells of *E. coli* in real time using the fluorescent fusion protein SeqA–YFP. However, monitoring plasmid transfer and assessing HGT frequencies in complex microbial communities in situ remain a challenge. Sørensen et al. (2005) published a critical review on HGT studies in situ. Direct evidence of the extent of in situ plasmid transfer in natural environments has been obtained by identification of plasmid-encoded phenotypes, such as antibiotic resistance or heavy-metal resistance, following the introduction of donor strains. This approach relies on the cointroduction of a marked recipient strain or the emergence of identifiable phenotypes among the indigenous bacterial populations (Sørensen et al. 2005). Plasmid transfer frequency in bulk environments such as bulk water and bulk soil is low (transconjugant/donor typically $<10^{-5}$). In many cases, transfer could only be detected after nutrient enrichment (Sørensen and Jensen 1998). This is in contrast to hot spots of bacterial metabolic activity and HGT, such as the rhizosphere and phylloplane of plants and other culturable surfaces, where transconjugant/donor ratios can be as high as 10^{-3} or even 10^{-1} for indigenous as well as introduced plasmids (Lilley et al. 1994; van Elsas and Bailey 2002).

Modern approaches to detect and quantify plasmid transfer use reporter gene technology. Due to simple detection by fluorescence microscopy, only fluorescence reporter genes have been used for in situ monitoring of HGT in natural environments. Biofilms are uniquely suited for HGT due to high bacterial density and metabolic activity even in the harshest environments (Wuertz 2002). Insights into the extent of HGT in biofilms were obtained from approaches combining fluorescently labeled plasmids and bacterial strains with confocal laser scanning microscopy and quantitative image analysis (Haagensen et al. 2002; Molin and Tolker-Nielsen 2003).

Christensen et al. (1998) investigated transfer of the TOL plasmid in flow-chamber biofilms of *P. putida*. Transconjugants were preferentially found on top of recipient microcolonies. Invasive transfer from new transconjugants to recipients in the microcolony was not observed. Online monitoring of transconjugant proliferation showed that the plasmid was primarily transferred vertically following a small number of HGT events (Sørensen et al. 2005).

The spatial structure of the biofilm has a decisive role in HGT. Bacterial conjugation was analyzed by observing the physical environment encountered by donor cells migrating into a biofilm matrix (Wuertz et al. 2004). Transconjugants were found deep inside biofilms grown in flow cells, indicating the ability of donor cells to penetrate beyond superficial surface layers (Sørensen et al. 2005).

Aspray et al. (2005) investigated conjugative plasmid transfer in a soil-based microbial biofilm in flow cells amended with 2,4-D. A 2,4-D-degrading donor strain, *P. putida* harboring a 2,4-D-catabolic conjugative plasmid tagged with *gfp* (pJP4::*gfp*) was inoculated into the flow-cell chambers containing 2-day-old biofilm communities. Transfer of pJP4::*gfp* from the donor to the bacterial community was detected by green fluorescence as monitored by confocal scanning laser microscopy (GFP fluorescence was repressed in the donor due to the presence of a chromosomally encoded *lacI*^{q} repressor gene). A 2,4-D-degrading transconjugant was isolated from the flow-cell chamber belonging to the genus *Burkholderia* (Aspray et al. 2005).

Conjugative plasmid transfer and plasmid mobilization in multispecies biofilms in continuously operated small-scale biofilm reactors have also been analyzed in the author's group. The biofilm communities consisted of different Gram-positive bacteria belonging to the genera *Staphylococcus* and *Enterococcus*. Plasmid transfer was monitored by GFP fluorescence. Donors and transconjugants were distinguished by an additional nontransferable fluorescence label in the donor cells. Transconjugants were obtained in intrageneric and intergeneric matings. Transfer rates were in the range of 10^{-8} transconjugants/recipient (Schiwon, K., Arends, K., and Grohmann, E., personal communication).

The use of GFP-tagged reporter plasmids for in situ studies has certain limitations, however. The fluorescence of GFP can be affected by environmental conditions such as high salt concentrations, low pH, and lack of oxygen, which is particularly relevant in dense biofilms. Expression of GFP in metabolically inactive or weakly active cells can be weak or even absent. Thus, expression cannot easily be distinguished from background fluorescence (Sørensen et al. 2005). Further efforts are required to develop more robust fluorescence labels with higher expression levels under environmental conditions.

7.7 Spread of Biodegradation Traits

Springael and Top (2004) published an excellent article on the state of the art of HGT in connection with microbial adaptation to xenobiotics. Characterization of bacteria that degrade organic xenobiotics has demonstrated that they can adapt to

these compounds by the expression of novel catabolic pathways. Some appear to have evolved by assembly of horizontally transferred genes, followed by mutations and gene rearrangements. New types of xenobiotic catabolic MGEs have been detected recently, the so-called catabolic genomic islands, which integrate into the chromosome after transfer (e.g., Toussaint et al. 2003; van der Meer and Sentchilo 2003). The presence of such xenobiotic-degrading bacteria in the biosphere has important environmental applications, such as the cleanup of polluted sites, fate of pollutants in the ecosystem, and their ecotoxicology (Head and Bailey 2003).

Van der Meer et al. (1998) provided a case of a potential in situ catabolic pathway assembly. They isolated chlorobenzene-mineralizing bacteria from chlorobenzene-contaminated groundwater. The isolates degraded chlorobenzene by a well-described two-step process (van der Meer et al. 1998). Springael and Top (2004) postulated that catabolic "precursor" genes might be present in the bacterial community before its exposure to contamination or they might be provided by "migrating" bacteria. Springael et al. (2002) showed that HGT resulting in the acquisition of xenobiotic degradation genes appears to occur with high frequency and over a relatively short period of time. Sentchilo et al. (2003) investigated the 105-kb genomic island of a *Pseudomonas* species carrying the *clcRABD* gene cluster that encodes the mineralization of chlorocatechols by a modified ortho-cleavage pathway. Induction of HGT of the *clc* element by 3-chlorobenzoate was demonstrated. Sentchilo et al. (2003) were the first to show that a pollutant can regulate transfer of the MGE encoding its metabolism.

Bathe et al. (2004) analyzed the possibility of enhancing degradation of 2,4-D in a sequencing batch biofilm reactor with the conjugative plasmid pJP4 encoding genes for 2,4-D degradation. Transconjugants were detected both by culture and culture-independent approaches in the 2,4-D-degrading biofilm. A 90% 2,4-D degradation was observed in the bioaugmented reactor within 40 h, whereas a control reactor without the plasmid contained 60% of the initial 2,4-D concentration after 90 h. This study showed the increase of 2,4-D degradation by conjugative transfer of pJP4 from an introduced donor strain to the bacterial community of a laboratory wastewater treatment system and demonstrated that adaptation of a microbial community to a xenobiotic compound can be accelerated by HGT of the respective catabolic genes (Bathe et al. 2004).

Bioaugmentation by HGT to mixed microbial populations in laboratory and pilot-scale sequencing batch biofilm reactors treating synthetic wastewater containing benzyl alcohol was analyzed by Venkata Mohan et al. (2009). A *P. putida* plasmid donor chromosomally labeled with the gene for the red fluorescent protein (RFP), harboring a GFP-tagged TOL plasmid that confers degradation of benzyl alcohol, was used. Survival of a bioaugmented strain, conjugative plasmid transfer, and increased degradation of benzyl alcohol were detected in the laboratory-scale reactor, but not in the pilot-scale reactor (Venkata Mohan et al. 2009).

7.8 Conclusions

Conjugative plasmid transfer is the most important means to disseminate resistance and catabolic genes among bacteria and to acquire them from other bacteria to cope with changes in the local environment. Many data have been collected in recent decades on the mechanism of HGT; protein key players have been identified and their enzymatic mechanisms elucidated. The three-dimensional structure of protein complexes required for horizontal plasmid spread has been solved for plasmids from Gram-negative bacteria, and detailed information on regulatory circuits involved in plasmid transfer of the sex-pheromone-responsive plasmids from Gram-positive enterococci has been obtained.

Ecology of HGT has kept pace with the advances in basic molecular and biochemical research. Research on in situ plasmid transfer has proceeded tremendously in recent decades, in particular due to the combined efforts of molecular biologists and microbial ecologists in the field. Experimental evidence for in situ plasmid transfer has been obtained for diverse aquatic and terrestrial habitats, biofilms on all kinds of surfaces, inner surfaces and organs of soil and water animals, habitats ranging from very oligotrophic to extremely nutrient-rich environments. Horizontal plasmid transfer appears to be a component of the natural lifestyle of all known bacteria.

This chapter summarizes the current knowledge of horizontal plasmid transfer in diverse environments with a focus on contaminated habitats, pointing out the contribution of microorganisms, in particular, of their mobilome (all transmissible or mobilizable genes), to the cleanup of polluted environments and the application of MGE in technical bioaugmentation processes to increase biodegradation of xenobiotic compounds.

7.9 Future Recommendations

Second-generation sequencing technologies have generated a large number of full-genome sequences including those of not only many pathogenic microorganisms but also numerous indigenous bacteria. Moreover, some research groups have focused on sequencing of whole plasmid genomes and genomes of other MGE of interest due to their prevalence in hospitals or in the environment. Determination of complete plasmid sequences from different origins with respect to host background and habitat will enable the comparison of numerous plasmid backbones and help decipher the evolution of mosaic structures of plasmid genomes, eventually leading to predictability of plasmid adaptation to environmental changes/challenges in the future.

Recent advances in fluorescence reporter technology (multiple labels, higher fluorescence intensities, fluorescence less affected by environmental conditions) and continuous improvements of microscopy techniques with three-dimensional resolution will facilitate the assessment of plasmid transfer efficiency in complex

environments. In conjunction with steadily improving and more realistic models of conjugative plasmid transfer and plasmid mobilization in complex microbial communities, they will help determine plasmid transfer frequencies in nature and presumably in the near future enable us to predict HGT events as responses to environmental stress.

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