REVIEW ARTICLE

Plasmids foster diversification and adaptation of bacterial populations in soil

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Abstract

It is increasingly being recognized that the transfer of conjugative plasmids across species boundaries plays a vital role in the adaptability of bacterial populations in soil. There are specific driving forces and constraints of plasmid transfer within bacterial communities in soils. Plasmid-mediated genetic variation allows bacteria to respond rapidly with adaptive responses to challenges such as irregular antibiotic or metal concentrations, or opportunities such as the utilization of xenobiotic compounds. Cultivation-independent detection and capture of plasmids from soil bacteria, and complete sequencing have provided new insights into the role and ecology of plasmids. Broad host range plasmids such as those belonging to IncP-1 transfer a wealth of accessory functions which are carried by similar plasmid backbones. Plasmids with a narrower host range can be more specifically adapted to particular species and often transfer genes which complement chromosomally encoded functions. Plasmids seem to be an ancient and successful strategy to ensure survival of a soil population in spatial and temporal heterogeneous conditions with various environmental stresses or opportunities that occur irregularly or as a novel challenge in soil.

Introduction

Horizontal gene transfer (HGT) is recognized as a major force contributing to bacterial diversification and adaptation (Iwasaki & Takagi, 2009). HGT can be mediated by different processes such as natural transformation, transduction or conjugation. The latter is assumed to be essential for a relatively rapid response to changing environmental conditions (Thomas & Nielsen, 2005). The contribution of each of the processes to horizontal transfer of plasmids among soil bacterial populations and the factors driving the transfer and proliferation of plasmid-containing bacteria in the soil environment are not fully understood. It is assumed that the importance of the different HGT processes will depend mainly on the characteristics of the bacterial species and of the mobile genetic elements (MGE) involved.

The process more specifically linked to plasmid acquisition is conjugation (Thomas & Nielsen, 2005). Conjugative plasmids are transmitted both vertically (segregation to daughter cells) and horizontally (transfer to another recipient cell). Conjugative transfer of plasmids to another recipient cell involves the transfer of the DNA as a nucleoprotein particle composed of a protein bound to the 5′ end of the single-stranded DNA from a donor to a physically attached recipient via a pore of the so-called conjugation apparatus (Zechner *et al.*, 2000; Varez-Martinez & Christie, 2009). Important components of the conjugative transfer are genes for the transfer and replication system, mating pair formation and the coupling protein, which are usually organized in operons (Frost, 2009; Smillie *et al.*, 2010).

Retrospective evidence for the potential role of plasmids in the diversification and adaptation of soil bacteria comes mainly from studying the presence and diversity of plasmids in soil isolates. Recently, quantitative or semi-quantitative data on the abundance of plasmid-related sequences in DNA directly extracted from soil provided information on the abundance of plasmids in soils (Smalla *et al.*, 2000b; Heuer *et al.*, 2009).

Although it is assumed that there is a link between environmental pollution and the abundance of plasmids,

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experimental data are scarce (Top *et al.*, 1995; Drønen *et al.*, 1998; Smalla *et al.*, 2006; Heuer *et al.*, 2009). The assumption is obviously based mainly on the observations that genes coding for enzymes that enable bacteria to resist antibiotics or heavy metal or to degrade xenobiotics are frequently carried on plasmids (Top & Springael, 2003; Smets & Barkay, 2005) and that plasmid-containing strains have often been isolated from polluted sites (Top *et al.*, 1995; Smit *et al.*, 1998; Gstalder *et al.*, 2003; de Lipthay *et al.*, 2008).

To provide a link between sequence-based insights into the potential role of plasmids in soil bacteria and the ecology of these plasmids in soil, the first part of this review is devoted to the intricacies of soil habitat, their constraints and the driving forces of plasmid-mediated gene transfer in the habitat. In the following sections, the methodologies used to study plasmids in soil bacteria and soil-related habitats such as the rhizosphere or the residuesphere will be examined. In addition, the factors triggering transfer as well as major constraints in our present understanding of plasmid-mediated gene transfer processes in soils will be unravelled. Sequence analysis of conjugative plasmids from soil bacteria has provided insights into their potential role in promoting genetic variation of populations so that they are better prepared for environmental stresses or provided with the opportunity to colonize empty niches. Insights into the common features and diversity of the plasmid backbone of selected plasmid groups with a broad or narrow host range as well as their accessory gene loads to the plasmid-driven adaptation of soil bacteria to pollutants, e.g. antibiotics, heavy metals and xenobiotics, will be discussed. In the last section of this review, evidence will be presented supporting our view that plasmids are an ancient and important way in which bacterial populations generate and maintain genetic variation.

Driving forces and constraints of HGT in soil

Soils are extremely diverse and complex habitats and they serve as sinks for many man-made xenobiotic wastes entering the soil, e.g. via sewage or manure. Soil bacteria not only drive major biogeochemical cycles (Kibblewhite *et al.*, 2008), they are also exposed to spatially heterogeneous selective pressure from man-made pollutants (Slater *et al.*, 2010). The architecture of the soil pore network formed by minerals, soil organic matter, fungal hyphae, roots, plant debris and the soil meso-fauna allows for the co-existence in close proximity of numerous micro-niches that vary in the presence or absence of water, soil pH, temperature, redox potential and nutrient sources, and pollutant concentrations (Young & Crawford, 2004). The heterogeneity of these various micro-habitats is likely to be the reason for the huge microbial diversity in soil, as the varying conditions influence the structural and functional composition of microbial colonizers (Nunan *et al.*, 2002, 2003; Zhou *et al.*, 2002). Although the number of bacterial cells per gram of soil can easily exceed 10^{10} and estimates of the numbers of different species range from 10^3 to 10^5 (Gans *et al.*, 2005; Schloss & Handelsman, 2006; Roesch *et al.*, 2007), only a rather small proportion of the soil surface is actually colonized by bacteria. Soil bacteria were shown to occur as micro-colonies, i.e. small agglomerates of cells that might be regarded as soil biofilms (Nunan *et al.*, 2001; van Elsas *et al.*, 2007). In view of HGT, the architecture of the soil pore network and in particular the pore space is of importance, as it strongly influences the nature and extent of the interactions between soil bacteria and the accessibility of nutrients and pollutants (Young & Ritz, 2000; Ranjard & Richaume, 2001).

But how frequent are HGT processes in soil? In general, it is assumed that transfer processes in soil occur rarely and will be selected over evolutionary time scales. In the soil environment the formation of aggregates or biofilms might contribute to an increased spread of conjugative plasmids in bacterial populations (Molin & Tolker-Nielsen, 2003). On the other hand, the high cell densities in these micro-colonies favour the cell-to-cell contact required for conjugal transfer of plasmids. In addition, the spatial isolation of these micro-colonies in soil is assumed to prevent plasmid-mediated gene transfer between populations that live in spatially distant micro-colonies, unless migrating cells can transfer plasmids between micro-colonies assisted by water flow. Furthermore, soils usually provide only limited nutrient resources, resulting in low metabolic activity of a large proportion of the soil bacteria.

Despite these particularities of soil, plasmids have been frequently detected in bacterial isolates from bulk or rhizosphere soils or in exogenously captured plasmids (Top *et al.*, 1995, 1996; Lilley & Bailey, 1997b; van Elsas *et al.*, 1998; Sobecky, 1999; Heuer & Smalla, 2007; Kuhn *et al.*, 2008; Pistorio *et al.*, 2008; Heuer *et al.*, 2009) or in DNA directly extracted from soil samples (Götz *et al.*, 1996; Smalla *et al.*, 2000b; Heuer *et al.*, 2002; van Overbeek *et al.*, 2002), which points to a major ecological function.

To explain the extant diversity of MGEs observed in soil bacteria despite potential constraints to plasmid transfer such as low metabolic activity or spatial isolation, the concept of 'hot spots' of HGT in soil was developed (van Elsas *et al.*, 2003). Typically, the rhizosphere, the residuesphere (decaying plant material) and the gut of soil arthropods, bacteriovorous nematodes or earthworms are seen as hot spots of HGT. In hot spots of HGT in soil, sufficient nutrients are assumed to increase metabolic activity of putative mating partners as well as the

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proliferation of transconjugants. Several studies on HGT with introduced bacteria carrying plasmids have shown that hot spots of HGT exist in soil (van Elsas *et al.*, 2003) and that nutrient availability is an important factor stimulating transfer events and the proliferation of resulting transconjugants. It is not clear yet whether selective pressure acts mainly via the provided fitness advantage to those bacteria with plasmids carrying resistance or degradative genes, or also significantly promotes gene mobility by induction of transfer genes, recombinase or integrase genes. In addition, these types of hot spots are assumed to occur also in micro-sites in soil, as in the complex heterogeneous pore network these sites might exist where bacteria have better access to substrates or pollutants adsorbed to clay particles or the soil organic matter. Micro-scale sampling techniques of specific bacterial taxa might be instrumental in providing a better understanding of HGT in soil (Slater *et al.*, 2010).

Strategies to detect and to capture conjugative plasmids from soil bacteria

To date, the knowledge on plasmids in soil bacteria is still biased towards those carried by taxa that are accessible through cultivation techniques. Furthermore, plasmids are also assumed to be present only in a small proportion of a given bacterial population and thus their abundance might often be below the detection limit. However, under the conditions of selective pressure they might become detectable. Figure 1 gives an overview of the experimental tool set available at present to study plasmids in soil bacteria. This scheme also contains recent metagenomics tools that have been already applied successfully for other

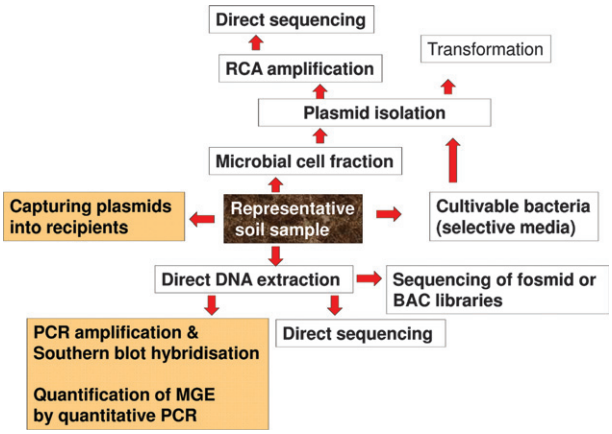

Fig. 1. Cultivation-dependent and -independent approaches to detect and analyze plasmids from soil bacteria. RCA: Rolling circle amplification; BAC: Bacterial artificial chromosome; MGE: Mobile genetic element.

environments such as sewage or contaminated river sediments, but not yet for soils. The advantages and potential limitations of the different methods are summarized in Table 1.

Direct detection of plasmid backbone genes in total community DNA was already suggested in the 1990s to obtain insights into the occurrence of conjugative plasmids in soil and related ecosystems, and PCR detection systems were provided for the known broad host range (BHR) plasmids at the time (Götz *et al.*, 1996). To increase the sensitivity and also confirm sequence specificity, PCR amplification was combined with Southern blot hybridization using probes generated from reference plasmids. The increasing number of newly sequenced plasmids revealed that primer systems designed on the basis of only a few sequences available at the time might fail to detect the extant diversity of different plasmid groups; for example, the originally proposed primer systems targeting the *trfA* gene of IncP-1 would not have efficiently amplified plasmid-specific sequences as well as the newly discovered groups of IncP-1γ, ε and δ from community DNA. More importantly, the *trfA* probes generated from the IncP-1α plasmid RP4 or the IncP-1β plasmid R751 would have failed to hybridize with plasmid DNA from IncP-1γ, ε and δ. This limitation needs to be taken into consideration when interpreting previously published studies (Heuer *et al.*, 2002; van Overbeek *et al.*, 2002; Smalla *et al.*, 2006; Binh *et al.*, 2008).

In the 1990s, several studies reported novel BHR plasmids based on the absence of hybridization with the Couturier replicon probes (Couturier *et al.*, 1988; Dahlberg *et al.*, 1998; Drønen *et al.*, 1998). The Couturier probe for IncP-1 would only have detected IncP-1α plasmids. The recently proposed new *trfA* primers (Bahl *et al.*, 2009) targeting IncP-1α, β, γ, ε and δ would have failed to detect the IncP-1ξ plasmids published by Norberg *et al.* (2011). If PCR amplicons are obtained from soil DNA, the diversity of PCR-amplified plasmid sequences can be analyzed by either cloning and sequencing or direct pyrosequencing. Although PCR-Southern blot detection is at best semi-quantitative, the rather high specificity of the probes enabled the assessment of the occurrence of different plasmid groups or subgroups.

Quantitative real-time PCR targeting plasmid-specific genes now provides for the first time the chance to quantify plasmids in total community DNA and to determine their relative abundance (plasmid copy number related to 16S rRNA gene copy number). PCR-based detection of plasmid-specific sequences can provide information on plasmid occurrence in soils and allows the correlation with plasmid abundance as well as plasmid-encoded functions and environmental pollution. The main limitation of PCR-based screening is that the genetic context of the

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Table 1. Methods for detection and characterization of plasmids in soil

Method	Advantage	Disadvantage
PCR-amplification from total community DNA by means of primers targeting sequences of the plasmid core (plasmid "backbone")	• Cultivation-independent • Increased sensitivity and specificity if combined with Southern blot hybridization • Quantification by real-time PCR • Diversity assessment by pyrosequencing or cloning and sequencing	• Bacterial hosts and accessory elements of the plasmids remain unknown • Limited detection of novel sequences
Exogenous plasmid isolation by bi- or triparental mating of selectable recipients and bacterial communities	• Independent from culturability of the original host • Sequencing allows a rather easy assembly, and characterization of accessory elements	• Depends on plasmid replication in the recipient, and presence and expression of selectable markers • Host remains unknown • Works mainly with gram-negative bacteria
Transformation of plasmid DNA directly extracted from microbial soil fraction by electroporation	• No optimization of transfer conditions • Plasmids from bacteria which are not metabolically active under the conditions of filter mating can be obtained	• Replication and expressed marker genes required • Method to extract plasmid DNA from the microbial soil fraction often influences the type (size) of plasmids
Pyrosequencing of plasmid DNA extracted from the microbial soil fraction	• Replication or presence of selectable markers not required • Discovery of novel plasmids replicating only in uncultured bacteria	• Rolling circle amplification steps required Bias towards small plasmids • Assembly of replicons difficult
Pyrosequencing of total genomic DNA, sequencing of fosmid or BAC libraries from environmental DNA	• Most comprehensive inventory • Independent of replication, or selectable markers	• Low abundance of plasmids relative to chromosomal fragments • Assembly of plasmids impossible or difficult • No information on hosts

plasmid-specific sequences amplified as well as of the host remain unknown. Furthermore, as shown above, the primers will only amplify target DNA which has no mismatches at the 3′ end.

To explore the diversity and the types of accessory genes carried, the plasmids need to be either isolated from the cultivable fraction or captured directly from soil bacteria into suitable recipients. Plasmid DNA could be isolated from resuspended bacterial colonies grown on selective media and transformed into suitable recipients. The capture of plasmids directly into recipients is termed exogenous plasmid isolation and enables plasmid isolation independently of the cultivability of their original hosts. Plasmids can be captured either in biparental or triparental matings (Bale *et al.*, 1988; Hill *et al.*, 1992; see Fig. 2a, b). Plasmid capturing in biparental matings is based on the ability of the plasmid to transfer to and stably replicate in a selectable recipient. Transconjugant selection is based on plasmid-mediated traits such as antibiotic or heavy metal resistance or on the basis of the ability to utilize xenobiotics as sole carbon compound. In triparental matings, plasmids are obtained from soil bacteria based on their ability to mobilize small mobilizable plasmids. Transconjugant selection is followed by the marker of mobilizable plasmid (e.g. IncQ plasmids). Although, based on their detection by PCR in total community-DNA, populations carrying IncP-1 plasmids are often not very abundant in soils, they are efficiently transferred in surface mating. Capturing frequencies were often higher from polluted sites (Top *et al.*, 1995; Drønen *et al.*, 1998; Smalla *et al.*, 2006) than from the corresponding non-polluted sites. Whether the increased transfer frequencies indeed reflect increased transferability or just an increased abundance of potential plasmid donors is still unclear.

New insights into plasmids from soil bacteria will come from the field of metagenomics. Pyrosequencing of DNA from rivers highly polluted with antibiotics revealed the presence of different plasmids and enabled the assembly of four small plasmids carrying different antibiotic resistance genes (Kristiansson *et al.*, 2011). The main advantage of the pyrosequencing of total or plasmid DNA extracted from soils is that novel plasmid sequences can be discovered. A major limitation is that the sequencing depth affects the detection of plasmids in less abundant populations and thus will cover only a fraction of the entire soil metagenome (Kristiansson *et al.*, 2011). Furthermore, assembly, particularly of large plasmids, will be difficult due to low copy or the use of random circle amplification.

Insights into plasmid transfer under soil conditions

The factors influencing plasmid transfer in bulk and rhizosphere soils were investigated with different plasmid groups in soil microcosm, greenhouse and field experiments.

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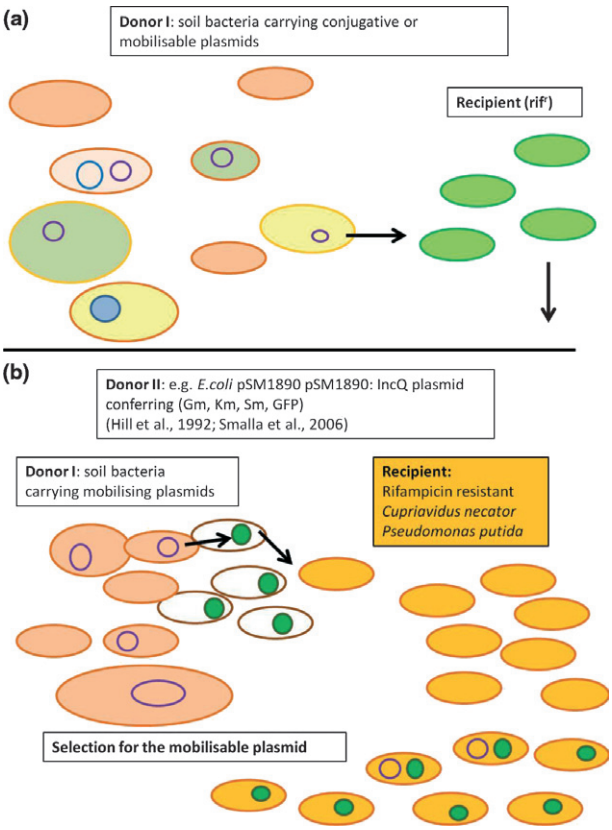

Fig. 2. Exogenous isolation of plasmids directly from soil bacteria into labeled recipients in (a) biparental and (b) triparental matings, modified from Bale *et al.* (1988) and Hill *et al.* (1992). Adapted by L. Bandounas.

Plasmid transfer was studied either by introducing strains carrying selectable plasmids or directly by following plasmid abundance and hosts in response to changing environmental factors. The main advantage of the introduction of strains carrying plasmids tagged with *gfp* or *rfp* gene under the control of a *lac* promoter, which is downregulated by the chromosomally located *lacI*$_q$, is that *gfp* is not expressed in the plasmid donor cell, but in transconjugant cells that do not contain *lacI*$_q$, allowing the detection of transconjugants at a single cell level (Dahlberg *et al.*, 1998). The detection in indigenous bacteria is unequivocally the result of a HGT process. When combined with flow cytometry and fluorescence *in situ* hybridization the range of hosts to which the plasmid is transferred can be determined (Musovic *et al.*, 2006).

Although direct detection of transconjugants in the soil matrix is still an experimental challenge, their detection along the roots and on the rhizoplane by means of confocal laser scanning microscopy has been successfully applied to localize transconjugants by Mølbak *et al.* (2003, 2007). These authors demonstrated transfer of the BHR plasmid pKJK5 (IncP-1ε) and the narrow host range plasmid pWW0 (IncP-9β) from *Pseudomonas putida* to indigenous bacteria on alfalfa sprouts (Mølbak *et al.*, 2003). Tagging of both the donor and the plasmids allowed a cultivation-independent quantification of both donors and transconjugants. Two orders of magnitude higher transfer frequencies were observed for pKJK5 as compared with the pWW0 plasmid (Mølbak *et al.*, 2003). Root growth and exudation patterns were demonstrated to influence the cell density and distribution patterns of donors and transconjugants (Mølbak *et al.*, 2007). Transconjugants were identified by plating and mainly belonged to *Enterobacteria* and *Pseudomonas*.

To determine the host range of pKJK5 in the rhizosphere of barley, Musovic *et al.* (2006) inoculated *P. putida* harbouring *lacI*$_q$ with a *gfp*-tagged pKJK5. Transconjugants were obtained after sorting by flow cytometry. The composition of putative transconjugants was determined by cloning and sequencing, documenting for the first time the conjugal transfer of IncP-1ε plasmids to gram-positive bacteria. As the transconjugants were not isolated, confirmation of the replication of IncP-1ε plasmids in *Arthrobacter* is still missing. *In situ* transconjugants could also be obtained using the tagged IncP-1 plasmids pEMT1 and pJP4 (mini Tn*5* Km*lacZ*). When transconjugants were selected based on their ability to utilize 2,4-dichlorophenoxyacetic acid (2,4-D), the transconjugants obtained belonged to a diverse range of *Burkholderia* (Goris *et al.*, 2002).

Alternative strategies for detecting transconjugants in soil were also used, e.g. with *Escherichia coli* introduced as plasmid donor (Pukall *et al.*, 1996): after cultivable *E. coli* cells were no longer retrieved, cultivable indigenous bacteria that received plasmids were detected by selective plating. The use of phages to kill the plasmid donor was another strategy used to detect indigenous transconjugants (Smit *et al.*, 1991). Although the species of the plasmid donor might influence which species will receive a plasmid (DeGelder *et al.*, 2005), this type of experiment clearly provides new insights into plasmid host range. There is plenty of evidence that a major factor influencing plasmid transfer frequencies is nutrients (Pukall *et al.*, 1996; Götz & Smalla, 1997). Nutrients from soil organic matter content, root exudates, manure or decaying compounds might increase the metabolic activity of donors and recipients, and alternatively stimulate the proliferation of transconjugants (van Elsas *et al.*, 2003).

The soil mobilome: insights from sequencing conjugative plasmids from soil bacteria

Genome-sequencing data and comparative genomics support the assumption that the horizontal acquisition of

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genetic modules is a major driver in bacterial evolution and adaptability (de la Cruz & Davies, 2000; Ochman *et al.*, 2000; Toussaint & Merlin, 2002; Smillie *et al.*, 2010). The increasing number of sequenced plasmids from soil also confirmed this assumption for plasmids from soil bacteria and allowed new insights into the diversity of 'plasmid backbone genes' which are involved in replication, maintenance and transfer of plasmids.

A major finding is the modular character of most of the plasmids. The sequences of the genes coding for replication, maintenance and transfer were shown to provide a far more reliable basis for plasmid grouping than the traditional incompatibility testing, enabling insights into the evolution of plasmids (Sevastsyanovich *et al.*, 2008; Smillie *et al.*, 2010). Sequencing of plasmids from soil bacterial isolates and exogenously isolated plasmids broadened our view of previously intensively studied and novel plasmid groups and the data suggest that plasmids are composed of core (backbone) genes and flexible (accessory) genes (Fig. 3).

The findings from sequence analysis and comparative genomics of selected BHR and narrow host range plasmid groups (for definition see Table 2) from soil bacteria will be discussed in the following paragraphs. In view of studies on the ecology of these plasmid groups, knowledge on the diversity of different plasmid backbone regions is an important prerequisite for the development of molecular detection systems and their use in the detection and quantification of the different plasmid groups in soil. The following section is focused on the plasmid backbone genes.

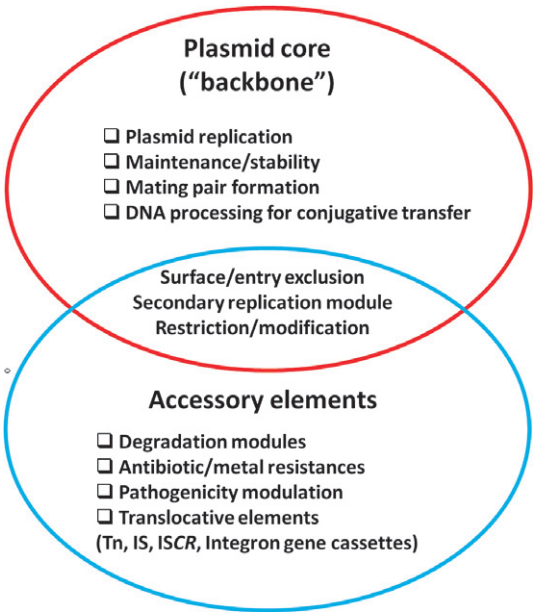

Fig. 3. Analysis of plasmid sequences indicated a modular structure composed of conserved core elements and flexible accessory elements.

Broad host plasmids in soil bacteria

Plasmids with a BHR are putatively important for many taxa in soil, as they can shuttle useful traits such as antibiotic-, heavy metal- or UV-resistance genes, efflux pumps, restriction modification systems, and toxin–antitoxin systems between different members of the bacterial communities in soil. Several studies have demonstrated that the transfer range of plasmids is often much broader than their replication range (Musovic *et al.*, 2006; Baharoglu *et al.*, 2010). Further retrospective evidence for this notion comes from sequenced genomes or plasmids of gram-positive and gram-negative bacteria that often carry identical antibiotic resistance genes or remnants of MGE (Tauch *et al.*, 2002a).

The ability of plasmids to replicate in a wide range of hosts requires that plasmid replication is to a large extent independent of the host's replication machinery. Furthermore, a tight regulation of plasmid-encoded genes ensures that the metabolic burden for their hosts is reduced. Repression of transfer genes might represent a compromise between the benefits for the plasmid to transfer horizontally and the associated metabolic burden on the hosts (Haft *et al.*, 2009). Repression of transfer genes might also reduce the susceptibility of the bacterial host to certain bacteriophages.

To predict the evolutionary range of hosts in which plasmids evolved, Suzuki *et al.* (2010) recently explored the use of nucleotide composition for a range of plasmids. Based on the assumption that plasmids acquire the genomic signature of their hosts, the trinucleotide composition of a range of plasmids was compared with all sequenced bacterial genomes. Indeed, the signatures of several typical BHR plasmids such as IncP-1, PromA and IncU displayed signatures that were dissimilar to any chromosomal signature, suggesting that these plasmids did not fix in a particular host (Suzuki *et al.*, 2010). Surprisingly, similar findings were also reported for IncP-9 plasmids, which are thought to have a much narrower host range.

An important feature of many conjugative BHR plasmids is that they efficiently mobilize or retro-mobilize smaller mobilizable plasmids. In fact, many conjugative BHR plasmids have been isolated based on their ability to mobilize IncQ plasmids (van Elsas *et al.*, 1998; Gstalder *et al.*, 2003). BHR plasmids most often reported from soil bacteria belonged either to the IncP-1 group or the recently suggested group of PromA plasmids (Van der Auwera *et al.*, 2009). Sequence-based insights into the diversity of backbone genes of these main groups of BHR plasmids in soil bacteria revealed a strong modular

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Table 2. Conjugative plasmids and translocative elements

Mobile genetic element	Properties
Broad host range plasmid	Self-transferable or mobilizable extrachromosomal replicon that is maintained in, and exchanges genetic information between a broad range of species; transferred functional modules are often located on translocative elements
Narrow host range plasmid	Transferable plasmid that is only maintained in closely related species
IS*CR*	Translocative element characterized by an IS91-like origin of replication and termination sites; probably transposes via a rolling-circle replication mechanism where occasionally 5′ sequences are co-transferred via misreading of the cognate termination site
Transposon	Translocative genetic element that can move within or between replicons by action of its transposase; flanked by inverted repeats; typically carries genes for antibiotic resistance or other phenotypes
IS-element	Translocative element that codes only for its transposase; multiple copies of the same IS-element promote genome plasticity by homologous recombination
Integron and gene cassettes	Integrons capture promoterless gene cassettes into an attachment site downstream of a promoter by action of its integrase, or translocate gene cassettes into other integrons or secondary sites

organization of the backbones, comprising genes for replication, maintenance and transfer and hot spots for acquisition of accessory genes. Whereas plasmids belonging to the IncP-1 group carry a large amount of accessory genes, plasmids of the recently proposed PromA group seem to promote bacterial adaptation by efficiently sampling the soil mobilome by retrotransfer (van Elsas *et al.*, 1998).

Plasmids belonging to the IncP-1 group

Plasmids belonging to the IncP-1 group have attracted the attention of plasmid biologists for more than 40 years, as these plasmids transfer and stably replicate in a wide range of bacterial hosts and are considered BHR plasmids (Adamczyk & Jagura-Burdzy, 2003). The first IncP-1 plasmids described were isolated from hospital specimens and thus IncP-1 plasmids were originally categorized as clinically important plasmids. Nowadays this plasmid group is considered ubiquitous and several studies using cultivation-independent methods have detected and isolated IncP-1 plasmids from soils and related ecosystems such as sewage and manure (Smalla *et al.*, 2000a, 2006; Heuer *et al.*, 2002; Bahl *et al.*, 2009).

Back in the 1990s, the complete sequence of the two archetype plasmids of the IncP-1α, RP4 (Pansegrau *et al.*, 1994) and the IncP-1β, R751 (Thorsted *et al.*, 1998) provided the first insights from comparative genomics. The sequence comparison confirmed the conservation of plasmid backbone sequences for replication, transfer and stable inheritance between the two subgroups. The plasmid backbone of both plasmids was found to be interrupted at two sites between *oriV* and *trfA*, and between the *tra* and *trb* operons (Thorsted *et al.*, 1998). In both plasmids, replication and transfer are globally regulated by the KorA and KorB protein encoded by genes of the central control operon. Nowadays, 28 published IncP-1 sequences are available. The sequenced plasmids originated from different geographic regions and environments, e.g. sewage, soils and river sediments. Based on 19 IncP-1 plasmids sequenced at the time, Schlüter *et al.* (2007) presented a comprehensive comparative genomic analysis of IncP-1 plasmids isolated from sewage. One of the most remarkable observations was the high nucleotide sequence conservation of the plasmid backbone. Based on DNA signature analysis, a more recent analysis of 25 complete backbone genomes of IncP-1 plasmids by means of newly developed bioinformatics tools demonstrated the existence of seven clades and the importance of homologous recombination as a key factor in IncP-1 plasmid backbone evolution. Genomic signature analysis data from Norberg *et al.* (2011) indicated that the different IncP-1 plasmids analyzed to date have adapted to hosts belonging to different species and that their backbones seem to originate from various parental plasmids.

Accessory elements were inserted at various sites of three backbone regions, the hot spots of insertion (Heuer *et al.*, 2004). In most of the IncP-1β plasmids, accessory genes are inserted in two specific regions between the *oriV* and the *trfA*, and between the *tra* and the *trb* operons. However, the IncP-1β plasmids pB2, pB3, pTP6 and pJP4 only carry insertions in one of the hot spots (Heuer *et al.*, 2004; Trefault *et al.*, 2004; Smalla *et al.*, 2006).

The most straightforward explanation of the evolutionary history of the completely sequenced IncP-1β plasmids is the existence of a common ancestor which was free of acquired genetic elements (Heuer *et al.*, 2004). This ancestor probably still exists in microbial communities and occasionally acquires accessory genes by transposition events. Indeed, recently, two cryptic IncP-1β plasmids were reported that do not carry any accessory elements, except two small open reading frames (ORFs) of

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unknown function: plasmids pA1 from *Sphingomonas* (Harada *et al.*, 2006) and pBP134 from *Bordetella pertussis* (Kamachi *et al.*, 2006).

Sota *et al.* (2007) determined experimentally that transposon insertions in the *oriV-trfA* and in the *tra-trb* regions of pB136 displayed a higher stability than in other regions. The authors suggested that the high structural similarity of IncP-1 plasmids might be due to a region-specific insertion combined with selection for stable and transferable plasmids.

Although IncP-1 plasmids were shown experimentally to have a wide host range, stable replication in the absence of selective pressure is strain-specific (DeGelder *et al.*, 2007; Heuer *et al.*, 2007; Sota *et al.*, 2010). Similarly, the permissiveness of genetically virtually identical *Dickeya* sp. isolates from a single field site for the uptake of IncP-1 plasmids differed by several orders of magnitude (Heuer *et al.*, 2010).

Sequencing also revealed that there are more subgroups of IncP-1 plasmids out there than previously assumed. Several exogenously isolated plasmids from soils have been assigned to novel subgroups such as IncP-1γ, IncP-1δ, IncP-1ε and the IncP-1ξ, that were previously only represented by one completely sequenced plasmid, pQKH54 (Haines *et al.*, 2006), pEST1044 (Vedler *et al.*, 2004) and pKJK5 (Bahl *et al.*, 2007), respectively.

Although Schlüter *et al.* (2007) discussed IncP-1ε as a tentative group, we have recently shown that plasmids belonging to this group could be frequently captured from manure (Binh *et al.*, 2008) and manure-treated soils (Heuer *et al.*, 2012). The data obtained by cultivation-independent methods point to an important role of the IncP-1ε subgroup in the agro-ecosystem, as all IncP-1ε plasmids captured from soil carry multiple antibiotic resistance and thus could easily enter the food chain via plant-associated bacteria.

Sequence analysis of three additional plasmids exogenously isolated from soil that were grouped based on their *trfA* sequence to the IncP-1ε group revealed that, except for their accessory genes, these plasmids were nearly identical to the previously sequenced plasmid pKJK5 that was also captured exogenously from agricultural soil in Denmark (Heuer *et al.*, 2012). Sequence comparison also showed that the primers developed by Götz *et al.* (1996) based on the sequences of RP4 and R751, were not optimal for amplification of the more recently discovered IncP-1 groups (Götz *et al.*, 1996).

New *trfA*-targeting primers were recently proposed by Bahl *et al.* (2009). The complete sequences of nine IncP-1 plasmids isolated from soil bacteria of various geographic origins revealed that IncP-1 plasmids are also important shuttles of beneficial traits in soil bacteria (Trefault *et al.*, 2004; Vedler *et al.*, 2004; Smalla *et al.*, 2006; Bahl *et al.*, 2007; Ma *et al.*, 2007). Table 3 lists these completely sequenced IncP-1 plasmids from soil, and 12 additional genomes from plasmids isolated from Norwegian agricultural soils were recently published (Sen *et al.*, 2011).

PromA-like plasmids

The complete sequence of plasmids pSB102 and pIPO2, both captured directly from rhizosphere bacteria by exogenous plasmid isolation, revealed striking similarities of the plasmids (Schneiker *et al.*, 2001; Tauch *et al.*,

Table 3. IncP-1 plasmids from soil with a complete published sequence

Plasmid (size)	Core	Source accessory genes	GenBank accession
pJP4 (87.7 kb)	β_{R751}	*Cupriavidus necator* JMP134, soil (Australia); 2,4-D and 3-chlorobenzoate degradation	AY365053
pADP-1 (108.8 kb)	β_{R751}	*Pseudomonas* sp. ADP from herbicide-contaminated agricultural soil (USA); atrazine degradation, mercury resistance	U66917
pAMMD1 (43.6 kb)	β_{R751}	Plant growth promoting biocontrol strain *Burkholderia ambifaria* AMMD from pea rhizosphere	CP000443
pA81 (98.2 kb)	β_{pB4}	*Achromobacter xylosoxidans* A8 from PCB-contaminated soil (Czech Republic); chlorobenzoic acids degradation, heavy metal resistance	AJ515144
pA1 (46.6 kb)	β_{pB4}	*Sphingomonas* sp. A1 from ditch soil (Japan); no accessory genes except two ORFs of unknown function	AB231906
pKJK5 (54.4 kb)	ε	Exogenously isolated from manured soil (Denmark); confers resistance to tetracycline and trimethoprim	AM261282
pEST4011 (77.0 kb)	δ	*Achromobacter xylosoxidans* EST4002 from agricultural soil (Estonia); 2,4-D catabolism	AY540995
pIJB1 (99.4 kb)	δ	*Burkholderia cepacia* 2a isolated from garden soil (UK); 2,4-D and malonate catabolism, mercury resistance	DQ065837
pAKD4 (56.8 kb)	δ	Exogenously isolated from mercury polluted soils (Norway); mercury resistance*	GQ983559

*Additional 12 IncP-1beta/epsilon plasmids (49–86 kb) from Norwegian agricultural soils were recently published (JN106164-75).

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2002b). Furthermore, the overall organization of the plasmids pIPO2 and pSB102 was very similar to plasmid pXF51 from the plant pathogen *Xylella fastidiosa*. Given the similarities between these plasmids, a novel family of BHR plasmids was proposed because both pIPO2 and pSB102 transferred to a wide range of gram-negative bacteria but did not belong to any of the known BHR plasmid groups revealed originally by PCR and probing, and confirmed by sequencing. Whereas plasmid pSB102 confers resistance to mercury compounds and was captured from rhizobacteria based on the acquired mercury resistance of *Sinorhizobium meliloti*, plasmid pIPO2 is a cryptic plasmid that was isolated due to its gene-mobilizing activity from wheat rhizobacteria. Comparative genomics based on their complete sequences enabled Tauch *et al.* (2002b) to discover that plasmids pSB102, pIPO2 and pXF51 had a strikingly similar overall genetic organization and led to the proposal of a new family of environmental BHR plasmids. The compact transfer region of all three plasmids is related to components of the transfer regions of the Ti and IncP-1 plasmids. However, the proposed mating pair formation system of all three plasmids strikingly displayed the highest homology to the type IV secretion system of the mammalian pathogen *Brucella* spp. (Schneiker *et al.*, 2001; Tauch *et al.*, 2002b). The replication and maintenance function possessed varying homologies to deduced protein sequences of different BHR plasmids.

The presence of long repetitive sequences is another interesting feature shared by pSB102, pIPO2 and pXF51. In pIPO2 and pSB102 at least one pair of inverted repeats was found flanking a varying number of different ORFs of presently unknown function (Tauch *et al.*, 2002b). Direct and inverted repeats promote rearrangements and thus contribute to the evolution of plasmids. The analysis of the complete genome sequence of *Collimonas fungivorans* led to the discovery of plasmid pTer331, which had a genetic organization very similar to that of plasmid pIPO2, and for many ORFs the deduced amino acid identity was above 90% (Mela *et al.*, 2008).

Recently, analysis of the complete sequence of plasmid pMOL98, a miniTn5-tagged derivative of pES1 isolated in a triparental mating from a hydrocarbon-polluted soil (Gstalder *et al.*, 2003), confirmed what was posited earlier, i.e. that pMOL98 is another member of the pIPO2-like plasmid family (Van der Auwera *et al.*, 2009).

Another candidate belonging to this group of plasmids, pMRAD02, was recently discovered in a sequencing project of *Methylobacterium radiotolerans* JCM2831 isolated from unpolished rice in Japan. All plasmids belonging to the pIPO2 family have in common the backbone modules comprising genes for replication, maintenance and transfer. The evolutionary relatedness of pIPO2, pTER331, pSB102, pMOL98 and pMRAD02 was recently assessed based on six concatenated backbone proteins (Van der Auwera *et al.*, 2009). Curiously, the plasmids captured in or isolated from *Betaproteobacteria* (plasmids pIPO2 and pMOL98 were both captured in a triparental mating in strains belonging to the genus *Cupriavidus*; pTER331 originated from *C. fungivorans*) were more similar to each other than to plasmids captured in or isolated from *Alphaproteobacteria* (pSB102 was captured in *S. meliloti*; pMRAD02 originated from *M. radiotolerans*).

Despite their overall highly similar organization, the sequences of the *repA* gene of pIPO2, pSP102 and pXF51 share only low nucleotide identities. The sequence divergence of the *repA* is certainly advantageous for the development of specific primer systems. Two primer sets targeting the *repA* gene of pIPO2 were used to determine the environmental distribution of replicons carrying this *rep* gene. Interestingly, PCR amplification from total community DNA in combination with Southern blot hybridization showed that the pIPO2 *repA* gene sequences were mainly detected in the rhizosphere of various crop plants and in previously planted agricultural soils (Tauch *et al.*, 2002b). However, to detect pIPO2-like plasmids primers it would be more advantageous to target sequences of the transfer module, as these genes are better conserved among the pIPO2-like plasmids. A high stability of pTer331 in *C. fungivorans* was observed during cultivation over 35 generations in liquid King's B medium, indicating an active stable partitioning system. Despite different experimental attempts to elucidate a putative role of pTer331, such as an improved rhizosphere competence, no phenotype trait except the ability to mobilize or retro-mobilize the IncQ plasmid pSM1890 could be attributed to the presence of the plasmid. Thus pIPO2, pTer331 and most likely pEES1 are cryptic plasmids, and it is assumed that these cryptic plasmids contribute to the adaptability of their hosts by promoting sampling of the soil mobilome (Mela *et al.*, 2008; Van der Auwera *et al.*, 2009).

The occurrence of these highly related plasmids in soils from various geographic regions and their ability to mobilize and retro-mobilize suggests an important role in plasmid-mediated adaptability of soil bacteria. Van der Auwera *et al.* (2009) proposed naming BHR-plasmids belonging to the pIPO2 family PromA in accordance with the traditional BHR-plasmids PromN (IncN), PromP (IncP-1), PromU (IncU) and PromW (IncW). Although plasmid nomenclature and classification will remain problematic, the increasing number of sequenced plasmid replicons will certainly facilitate a more reliable classification and, even more importantly, provide the tools for studying their ecology and importance in adaptation of soil bacterial populations to changing environments.

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Conjugative narrow host range plasmids from soil bacteria

Conjugative plasmids that stably replicate only in a restricted number of taxonomically related species or those that are found only in a limited number of hosts, are here termed narrow host range plasmids. Baharoglu *et al.* (2010) speculated that such plasmids generally have the strategy to inhibit the SOS response to conjugative entry of ssDNA in their natural host, by the action of specialized anti-SOS genes. In contrast, such special adaptations to a particular host are not useful for BHR plasmids, which require host-independent mechanisms of cost reduction. For the same reason, narrow host range plasmids might tend to transfer modules which require more integration into cellular networks for their function. For example, plasmid-transferred genes amending upper degradative pathways of aromatic compounds are only beneficial for particular hosts with a corresponding lower pathway.

IncP-9 plasmids

Plasmids belonging to the IncP-9 group were most often detected in *Pseudomonas* isolates from polluted soils and are supposed to play an important role in the adaptation of *Pseudomonas* populations. In contrast to IncP-1 plasmids their biology is far less studied and the host range of IncP-9 plasmids seems to be narrow, as no transfer to recipients other than *Pseudomonas* has been shown (Krasowiak *et al.*, 2002). At present, the complete sequences of four IncP-9 plasmids, pWW0 (Greated *et al.*, 2002), pDTG1 (Dennis & Zylstra, 2004), pNAH20 (Heinaru *et al.*, 2009) and pNAH7 (Sota *et al.*, 2006), are available, revealing an approximately 35-kb IncP-9 core, with genes involved in replication (*oriV*, *rep*), partitioning (*par*, *res*) and transfer (*mpf*, *tra*).

Based on the sequences of *oriV* and *rep*, nine subgroups of the IncP-9 group were recently proposed (Sevastsyanovich *et al.*, 2008). PCR, sequencing and hybridization were used to explore the phylogenetic relatedness and sites of accessory DNA of 30 different plasmids previously affiliated to IncP-9. Overwhelmingly, IncP-9 plasmids analyzed in this study were isolated from polluted soils from different states of the former Soviet Union. The geographic bias reflects the collaborative research activities of groups in Pushino, Minsk and Birmingham (Sevastsyanovich *et al.*, 2008). PCR amplicons obtained with primers targeting the *oriV* and the *rep* region (398 bp) were obtained for all but two plasmids previously identified as IncP-9.

Based on the sequence divergence of *oriV* and *rep*, the 30 plasmids analyzed can be divided into two major branches (pWW0-like and pDGT1-like, respectively) (Sevastsyanovich *et al.*, 2008). These data confirm previous results by Izmalkova *et al.* (2006). However, it is important to note that the DNA sequence identity of IncP-9 *rep* genes varies by only up to 7%, in contrast to the high sequence divergence of the replication-initiating protein of IncP-1 plasmids, which differ by 22–29% with up to 16% divergence in the IncP-1β subgroup (Sevastsyanovich *et al.*, 2008). From this comparison the nine subgroups suggested for IncP-9 plasmids could be considered one group. On the basis of the *oriV* and *rep* sequences of 28 IncP-9 plasmids, a novel primer system was recently developed and applied to detect IncP-9 plasmids in total community DNA in soils from various geographic regions (G. C. Ding & K. Smalla, personal communication). Cloning and sequencing of PCR amplicons revealed a surprisingly high diversity of IncP-9 amplicons, suggesting that, similar to IncP-1 plasmids, various subgroups of IncP-9 plasmids might co-occur in the same environmental niche and contribute to a rapid adaptability of *Pseudomonas* populations.

Low GC plasmids

Studies on the effect of veterinary medicine introduced into soil via manure on the abundance of transferable antibiotic resistance recently led to the discovery of another novel plasmid group. Plasmids belonging to this group were the plasmids most frequently captured directly from manure-treated soil in *E. coli* in several independent experiments. These plasmids did not hybridize with any of the previously described plasmid probes or primers and displayed a huge diversity, based on their plasmid restriction patterns and the antibiotic resistances conferred (Binh *et al.*, 2008; Heuer *et al.*, 2009).

The complete sequences of three representatives of this group – pHHV35, pHHV216 and pHH1107 – were recently determined and showed that, surprisingly, all three plasmids shared virtually an identical plasmid backbone of approximately 30 kb and had an unusually low GC content of, on average, 36%. As with many other plasmids, the plasmid backbone had a modular structure and comprised ORFs with replication, maintenance and transfer functions. Also the conjugation machinery of the LowCC plasmids is related to other type IV secretion systems and the deduced amino acid sequence of the transfer genes displayed moderate homology to the pIPO2/pSB102/pTer331 family (up to 46% amino acid identity in *traB*). Interestingly, the replication module (*oriV*, *rep*) had 5% lower GC content than the transfer and maintenance modules and was clearly of different descent, with the closest hits to *Acinetobacter baumannii*. The accessory regions of pHHV35, pHHV216 and pHH1107 had a mosaic structure with multiple antibiotic resistance genes

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and were similar in size (27, 28.3, and 28 kb, respectively; see Fig. 4).

Based on the sequence data, PCR primers were developed to target *repA* and *traN* gene, facilitating both detection of LowCC-type plasmids in soil bacteria or transconjugants and the quantitative detection of their abundance by real-time PCR (Heuer *et al.*, 2009). Real-time PCR revealed an astonishingly high abundance of these replicons in several soils; considering the seemingly huge diversity of their accessory gene load, Heuer *et al.* (2009) concluded that this plasmid group plays an important role in disseminating antibiotic resistance genes among soil bacterial population.

Plasmids of the *B. cereus* group

Recent whole genome sequencing projects of several isolates of the *Bacillus cereus* group revealed that the isolates previously classified as *B. cereus*, *Bacillus thuringiensis* or *Bacillus anthracis* are only distinguished by the content of plasmids and their accessory genes. Plasmid-encoded toxins are responsible for the deadly anthrax disease caused by *B. anthracis* and the plasmid-encoded insecticidal δ-endotoxins (*cry* genes) cause toxicity in insects. Genes carried by plasmids pXO1 and pXO2 (Okinaka *et al.*, 1999) encode the set of virulence factors characteristic for *B. anthracis*, the tripartite toxin and the poly-γ-glutamate capsule. The availability of the pXO1 and pXO2 plasmid sequences also provided the basis for the development of PCR primer systems targeting different plasmid backbone genes and genes coding for toxins. The application of these primer systems for screening environmental isolates belonging to the *B. cereus* group provided evidence that pXO1- and pXO2-like replicons can be detected in *B. cereus* isolates from soils (Hu *et al.*, 2009a, b).

The presence of pXO1 and pXO2 plasmids in a collection of 1000 isolates originating from various habitats and different geographic regions was analyzed by means of PCR primer systems targeting *repA* (pXO1-like), *repX* (pXO2-like), *virB4*, *virB11* or *virD4*. Whereas 6.6% and

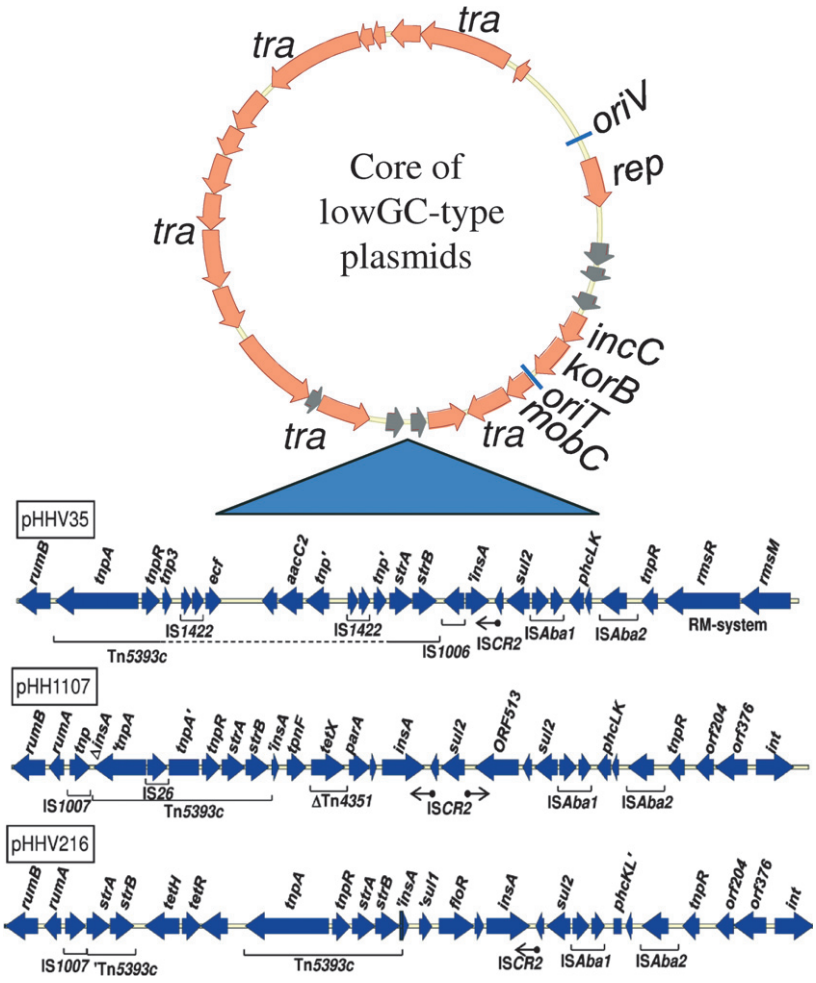

Fig. 4. LowGC plasmids share identical backbone genes but vary considerably in their flexible genes. Modified from Heuer *et al.* (2009).

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7.7% contained *repA* and *repX*, respectively, only 1.5% contained the *tra* genes (pXO2 plasmids only). However, none of the plasmids carried the toxin and capsule genes of *B. anthracis*.

In this study, pXO1- and pXO2-like plasmids were also detected for the first time in *Bacillus mycoides*. Again, self-transfer and mobilizing activity was observed among pXO2 plasmids carrying the *tra* gene. Interestingly, components (VirB4, VirB11, VirD4) of the T4SS-like transfer system of gram-negative bacteria were found encoded on several pXO2-like plasmids, suggesting that the three proteins also form a fundamental core in gram-positive bacteria (Hu *et al.*, 2009b).

A model for a T4SS-like transfer system was also recently proposed for the streptococcal broad host range plasmid pIP501 based on a comprehensive protein–protein interaction mapping of all pIP501-encoded Tra proteins by means of yeast two-hybrid assays (Abajy *et al.*, 2007). Combination of multi-locus sequence type (MLST) characterization of 80 *B. cereus* isolates from soil and PCR screening for the presence of pXO1 or pXO2 (Hu *et al.*, 2009a) showed that strains with identical genomic background based on MLST carried different plasmid types, indicating substantial diversification by plasmid transfer. However, among the collection of pXO1- and pXO2-carrying isolates investigated in this study, only four pXO2 plasmids carried the main components of the *vir* transfer system and only one *vir*-positive isolate contained a conjugative plasmid that was also capable of mobilizing the small not self-transferable plasmids pUB110 or pBC16.

The presence of anthrax toxin and the capsule gene were also tested in all isolates that carried pXO1 or pXO2 by PCR but none of the isolates was positive. Conjugation, mobilization and also retro-mobilization were recently shown for plasmid pXO16 from *B. thuringiensis* (Timmery *et al.*, 2009). The 350-kb plasmid conferring an aggregation phenotype secretion was shown not only to conjugate efficiently, but also mobilize and retro-mobilize. Thus plasmid capturing might be an important trait that facilitates the sampling of the horizontal gene pool.

Successful colonization of niches and adaptation to changing environments through horizontally transferred MGEs

Horizontally transferred genes can persist for a long time in a fraction of a bacterial population even if they are slightly deleterious (Novozhilov *et al.*, 2005). This might be especially true for soil, where growth rates are rather low and the putative horizontal gene pool is large due to the enormous bacterial diversity. Thereby MGEs create standing genetic variation, by which adaptation to a rapid environmental change or habitat expansion are more likely to be achieved than by new mutations (Jain *et al.*, 2003; Hermisson & Pennings, 2005).

In the previous section we discussed the insights gained from sequence analysis and comparative genomics of various plasmids of soil bacteria, and the sequence-based development of primer systems targeting plasmid backbone genes to allow the detection of particular plasmid groups in environmental settings. In the following paragraphs we will focus on the accessory genes on these plasmid groups that contribute to the adaptation of soil bacteria.

It is assumed that the so-called accessory gene load that often differs greatly among plasmids with identical plasmid backbones, gives an advantage to cells carrying these plasmids under the conditions of exposure to contaminants such as antibiotics, heavy metals and xenobiotics (Dennis, 2005; Schlüter *et al.*, 2007; Heuer *et al.*, 2009). The heterogeneous distribution of these compounds in soil is thought to contribute to spatially heterogeneous selection pressure on bacterial populations at small scales (Slater *et al.*, 2010).

Adaptation to antibiotics in soil

Many bacteria and fungi in soil have the ability to produce low molecular mass compounds which have antibiotic activity. For a long time it was assumed that the primary role of these compounds was in the competition among soil bacteria, but recent studies by means of transcriptomics have shown that antibiotics at sub-inhibitory concentrations modulate gene expression or function as signalling molecules (reviewed by Davies *et al.*, 2006; Martínez *et al.*, 2009). The effects of antibiotics at sub-inhibitory concentrations on a range of cellular responses were recently shown to differ among different classes of antibiotics. Thus the present view on antibiotics produced by soil bacteria is that, besides their putative role in competition, antibiotics at sub-inhibitory concentration are important signalling molecules in complex networks that are at present poorly understood (reviewed by Aminov, 2009). In contrast to the wide range of effects observed on the physiology of bacterial cells, the response to sub-inhibitory concentrations of antibiotics was frequently found to result in enhanced antibiotic gene transfer frequencies and often co-selection of various antibiotic resistance genes, which were genetically linked through their localization on the same MGE. SOS-responses induced by antibiotics such as fluoroquinolones were shown to increase HGT more than 300-fold (Beaber *et al.*, 2004). Based on the observation that sub-inhibitory concentrations of antibiotics accelerate genetic variability and HGT as reported in several studies, Aminov (2009) suggested

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that this mechanism might have been selected in the course of evolution in order to explore new niches. Antibiotic resistance genes were detected in soil bacteria that were most likely unaffected by the anthropogenic use of antibiotics (Riesenfeld *et al.*, 2004; D'Costa *et al.*, 2007; Demanèche *et al.*, 2008). This is not too surprising as more than 80% of the antibiotics presently used in hospitals originate from soil bacteria either as their natural product or as semi-synthetic derivatives. The presence of resistance genes providing self-protection in antibiotic-producing strains and in so-called non-producers is very well documented. Several cases where identical resistance genes were found in producing and non-producing *Streptomyces* strains have been described (Laskaris *et al.*, 2010), suggesting the involvement of HGT.

In soil bacteria the production of antibiotics is highly regulated, likely at sub-inhibitory levels. In contrast, the concentrations of antibiotics used in human and veterinary medicines or in plant protection are significantly higher (Martínez, 2008). The consequences of the large-scale use and environmental release of antibiotics on soil microbial communities are difficult to predict. The fate of antibiotics introduced into soil via manure or surface water depends very much on the group of antibiotics. The shape of the selection landscape is highly variable in space and time (Turner *et al.*, 2002). Often a rapid decline of the $CaCl_2$ extractable fraction is observed, and for a long time it was assumed that rapidly sequestered antibiotics might not impact soil bacteria. However, based on the recent findings concerning the unexpected manifold activities of antibiotics at sub-inhibitory concentrations the question of whether sequestered antibiotics might still influence the transcriptome of soil bacteria or influence transferability of plasmids, needs further attention.

Although there are many survey studies that aimed to correlate the abundance of antibiotic resistance genes and pollution with antibiotics, correlations were often problematic, as many different factors may have influenced the relative abundance of antibiotic resistance genes in soil bacterial populations (Heuer *et al.*, 2002; van Overbeek *et al.*, 2002; Byrne-Bailey *et al.*, 2009). Most importantly, what usually remains unclear is whether an increased abundance of antibiotic resistance is the result of the propagation of existing populations carrying these antibiotic resistance genes (vertical transmission) or whether it is due to horizontally spread antibiotic resistances. According to many retrospective studies, the wide use of antibiotics in veterinary and human medicine seemed to foster the mobility of antibiotic resistance genes in soil (Tschäpe, 1994; Witte, 1998; Heuer *et al.*, 2009). The ability of IncP-1 plasmids to efficiently transfer in soils deserves particular interest in view of their exceptional BHR, and the recently discovered occurrence of plasmids belonging to the IncP-1ε group that seem to play an important role in the dissemination of antibiotic resistances in the agro-ecosystem.

To assess the effect of sulfadiazine introduced into soil via manure on the abundance of sulfadiazine resistance genes (*sul1*, *sul2*, *sul3*) and their transferability, soil microcosm experiments were performed with two soil types. The abundance of *sul1* and *sul2* genes in total community DNA determined by quantitative PCR was significantly increased when soil was treated with manure spiked with sulfadiazine even 2 months after manure application. Also plasmid capturing directly from soil slurry into an *E. coli* strain was found to be significantly increased in soils treated with manure spiked with sulfadiazine compared with soils treated with non-spiked manure. Among the plasmids captured directly from soil bacteria into *E. coli*, sulfadiazine-resistance plasmids belonging to the LowCC-type plasmids were the most frequently encountered (Heuer *et al.*, 2009). Although these plasmids were isolated based on their ability to confer sulfadiazine resistance to their *E. coli* recipient strain, they encoded resistance to numerous different antibiotics. Sequence analysis of three representative plasmids – pHHV35, pHHV216 and pHH1107 – revealed that they differed only in their accessory gene load. The accessory region of all three plasmids had a similar size of approximately 28 kb and an average G+C content of 47% (compared with 37% G+C content of the plasmid backbone). Although similar antibiotic resistance genes (*sul2*, *strAB*,) and MGEs (IS, IS*CR*2, Tn*5393*) were found on the accessory gene modules, they strongly differed in their structure, and the mosaic structure of the accessory regions was obviously shaped through extensive HGT processes.

The sequence of plasmid pHH1107 provides a good example that the use of sulfadiazine in animal husbandries would co-select for instance resistance towards all kinds of tetracyclines, including the third generation tigemycin due to the physical linkage of *tetX* on the same plasmid. Using [3H]leucine incorporation during bacterial growth Brandt *et al.* (2009) demonstrated a rapid pollution-induced bacterial community tolerance to sulfadiazine in soil hot spots amended with artificial root exudates. These authors suggested that the rhizosphere or manure-amended soil should be regarded as a hot spot for the proliferation of bacteria resistant or tolerant to antibiotics. The observed increased community tolerance towards sulfadiazine in the presence of artificial root exudates most likely reflects the adaptation of soil bacteria fostered by horizontally transferred MGE, as the importance of nutrient availability for transfer of conjugative or mobilizable elements in soil is very well documented (Pukall *et al.*, 1996; Götz & Smalla, 1997). Among these

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plasmid groups, most likely the IncP-1ε and the LowGC plasmids play an important role in disseminating antibiotic resistance genes in soil.

Adaptation to metal pollution in soil

Many soils contain metal pollution of either anthropogenic or pedogenic origin. Metal pollutions are typically heterogeneously distributed in the soil matrix and their bioavailability depends on various biotic and abiotic factors. Man-made mercury contamination of soils and sediments is a serious environmental problem due to its high toxicity even at very low concentrations. Thus it is not too surprising that plasmids from soil isolates or exogenously captured carry often mercury resistance genes. The abundance of mercury resistance in soil or sediment bacteria has often been found to be correlated with mercury pollution (Drønen *et al.*, 1998; Smit *et al.*, 1998; Smalla & Heuer, 2006; de Lipthay *et al.*, 2008). However, plasmids exogenously captured from the phytosphere of sugar beet or the rhizosphere of alfalfa also often carried mercury resistance transposons (Lilley & Bailey, 1997b; Schneiker *et al.*, 2001). Mercury resistance plasmids obtained from the sugar beet rhizosphere were large (more than 300 kb) and had a narrow host range confined to *Pseudomonas*. The sequence of plasmid pQBR103 revealed very little homology of this plasmid to any known replicon. The presence of pQBR103 reduced the fitness of the inoculant strain *Pseudomonas fluorescens* SBW25 in the phytosphere of young sugar beet plants under greenhouse and field conditions (Lilley & Bailey, 1997a). However, the relative fitness of plasmid-carrying strains dramatically increased in maturing sugar beet plants, suggesting that plasmid-encoded traits might confer benefits that improve the survival and colonization of *P. fluorescens* SBW25 in the maturing sugar beet phytosphere. This conclusion was also supported by the observation that these plasmids were captured *in situ* in *P. fluorescens* SBW25 typically at a mid- to later period of plant development (Lilley & Bailey, 1997b).

Recently, Slater *et al.* (2008) investigated the effects of uniform or spatially heterogeneous $HgCl_2$ pollution on selection of *P. fluorescens* SBW25 carrying the mercury resistance plasmid pQBR103 compared to the plasmid-free strain. Mercury pollution resulted in an increased fitness of the mercury-resistant population relative to the sensitive population but, more importantly, spatially heterogeneous mercury pollution resulted in negative frequency-dependent selection of the resistant population, meaning that when Hg^r cells were rare, their growth advantage compared with that of sensitive cells was high, whereas when they were common, there was little advantage to being resistant.

In soils where spatially heterogeneous mercury pollution will prevail, it can be assumed that plasmid-carrying and plasmid-free populations might coexist. Different studies suggested that IncP-1 plasmids might play an important role in the adaptation of microbial communities in surface and subsurface soils exposed to mercury pollution (Smalla *et al.*, 2006; de Lipthay *et al.*, 2008). In soil microcosm experiments with surface and subsurface soil exposed to mercury, an increased abundance of both IncP-1 *trfA* and *merA* was observed compared with the control, which could result from the proliferation of existing IncP-1 plasmid-containing populations or from HGT (de Lipthay *et al.*, 2008). Transposon-located mercury resistance genes were identified on several IncP-1 plasmids such as pJP4, pADP1 and pIJB1. The complete nucleotide sequence of the IncP-1β plasmid pCNB1 from the *Comamonas* strain CNB-1 carried resistance genes to arsenate and chromate (Ma *et al.*, 2007). Although this strain was originally isolated from activated sludge, it efficiently colonized the rhizosphere of alfalfa (Liu *et al.*, 2007).

Mobilization of IncQ plasmids carrying the *czc* (pMOL187) or the *ncc* (pMOL222) cassettes and long-term retention of the introduced plasmids was shown by Smets *et al.* (2003) in metal-stressed subsurface soils under low carbon flux conditions. However, mobilization was dependent on the joint introduction of the mobilizing IncP-1α plasmid (RP4), indicating a low mobilizing capacity of the bacterial community in subsurface soil. At high cadmium concentrations the plasmid transfer or retention was found to be enhanced (Smets *et al.*, 2003).

An outstanding example for the adaptation of a soil bacterium to strong selective pressure by a range of heavy metals is strain CH34, which was isolated in the 1970s from a metallurgical site in Belgium with high levels of bioavailable heavy metals. It has since been studied in great detail by Max Mergeay's group. The strain CH34 was originally termed *Alcaligenes eutrophus*, later *Ralstonia metallidurans* and today *Cupriavidus metallidurans*. Whole genome sequencing revealed that the genetic determinants for heavy metal resistance in *C. metallidurans* were mainly located on chromosome 2 and on the two megaplasmids pMOL28 and pMOL30, and at least 11 islands were identified (van Houdt *et al.*, 2009). Resistance of *C. metallidurans* towards inorganic and organic mercury compounds was achieved by introducing the IncP-1β plasmid pTP6, which carries the complex mercury transposon Tn*5058* (Rojas *et al.*, 2011).

Adaptation to xenobiotic pollution in soil

Plasmid-mediated gene transfer is also assumed to play a major role in bacterial adaptation to xenobiotics (Top & Springael, 2003). Xenobiotics are defined as man-made compounds and before their introduction into the

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environment, bacterial populations were not exposed to them. Thus the evolution of plasmids carrying degradative genes for xenobiotics must have occurred rather recently.

Sequence analysis of numerous catabolic plasmids originating from soil isolates or exogenously captured from soil bacterial communities into recipient strains have improved our understanding of how these catabolic plasmids evolved. Comparative sequence analysis revealed that similar plasmid backbones carry various different degradative genes (Dennis, 2005). Experimental evidence and sequence data suggest that transposition, homologous and illegitimate recombination, gene duplication, and inversion must have contributed to the evolution of the catabolic genes present in the different plasmids analyzed so far (Dennis & Zylstra, 2004; Dennis, 2005; Larraín-Linton *et al.*, 2006). The IncP-9 plasmid pDTG1 has a remarkably different G +C content for genes encoding the upper pathway for the degradation of naphthalene to salicylate (52.7%) and those coding for the lower degradation pathway (61%), strongly suggesting that at least the genes coding for the upper degradation pathway originated from other organisms than Pseudomonads (Dennis & Zylstra, 2004).

Mutations resulting in changes of expression levels or gene loss were also shown to be important processes in the amelioration of plasmids to their hosts (Dahlberg & Chao, 2003; Sota *et al.*, 2010). The more frequent isolation of catabolic plasmids from polluted sites can result from vertical transmission of existing plasmids occurring in a few cells of a population (proliferation of plasmid-containing population) or from horizontal transfer. While catabolic operons involved in the degradation of various xenobiotics such as atrazine, 3- chlorobenzoate, 2,4-D, 4-chloronitrobenzene and 3-chloroaniline (Dejonghe *et al.*, 2002) were reported on IncP-1 plasmids (see Table 1), IncP-9 plasmids isolated from various polluted soils conferred mainly degradation of naphthalene, phenanthrene, anthracene, toluene, xylene and salicylic acids. Interestingly, IncP-9 plasmids conferred either degradative capacity or antibiotic resistances. Similar genes involved in naphthalene degradation occur on different IncP-9 and IncP-7 plasmids. The host population of both plasmids is *Pseudomonas* and it is suggested that both plasmids may have spent some time in the same cells and that transposases or the phage integrase activity might have contributed to the assembly of degradative genes (Dennis, 2005).

Carriage of MGE: a burden or a benefit to soil bacterial populations?

Transferable plasmids most likely cannot survive as purely parasitic elements or by only temporarily providing beneficial traits to their host (Bergstrom *et al.*, 2000). After horizontal transfer of a plasmid, it will probably not fit well into the cellular network and the ecological context of the new host. So, first of all, the plasmid is a burden for the recipient under regular environmental conditions (Fig. 5). If the plasmid encodes beneficial traits, then its chromosomal integration followed by loss of the plasmid will provide a selective advantage to the host because this eliminates the cost associated with plasmid maintenance and transfer (Fig. 5). The chromosomal integration is often facilitated by translocative elements such as transposons, which carry functional elements between plasmids and chromosomes, so that plasmid-borne beneficial functions may spread within a population without the need to maintain the plasmid. Plasmid-bearing cells will be out-competed by plasmid-free relatives if the cost of carrying the plasmid backbone is not balanced by a benefit for the cell. However, in most cases foreign DNA will be rather deleterious than increase the fitness of the recipient (Kurland, 2005; Hao & Golding, 2006). Therefore, the majority of acquired genes do not persist in evolutionary lineages of soil bacteria, which typically have large effective population sizes so that selection efficiently removes non-beneficial acquired genes (Ochman & Davalos, 2006). As a protective measure against deleterious gene acquisition, many cellular mechanisms limit the transfer, uptake and stabilization of foreign DNA in bacteria (Thomas & Nielsen, 2005).

Nevertheless, MGEs are abundant in soil bacterial populations. The incidence of plasmids in bacterial isolates from diverse habitats was reported to range from nearly 100% to < 10% (Sobecky, 1999; Cook *et al.*, 2001; Malik *et al.*, 2002; Thomas, 2004). The incidence and diversity of conjugative pSmeSM11a-like plasmids in *S. meliloti* was analyzed by Kuhn *et al.* (2008). Five of 21 strains isolated from one field and four of 16 strains from another field contained plasmids with pSmeSM11a-like backbone genes. Most plasmids differed in size and accessory genes. In an earlier field experiment, where a genetically marked Pseudomonad was established in the sugar beet phytosphere, diversification by acquisition of naturally occurring conjugative plasmids could be shown (Lilley & Bailey, 1997b).

So why are these plasmids maintained in bacterial populations? Sometimes, transferable plasmids even seem to carry nothing but the core genes for transfer and maintenance (Heuer *et al.*, 2004) or only minor accessory elements such as the broad host range IncP-1 plasmids pA1 and pBP136 (Harada *et al.*, 2006; Kamachi *et al.*, 2006). The long-term existence of cryptic plasmids could be explained if bacterial populations actively maintain a type of plasmid backbone, regardless of accessory elements. As transferable plasmids are highly flexible in their modular structure and typically present in only a fraction of a population, they are ideal tools to create standing genetic variation within populations. This is by far more efficient

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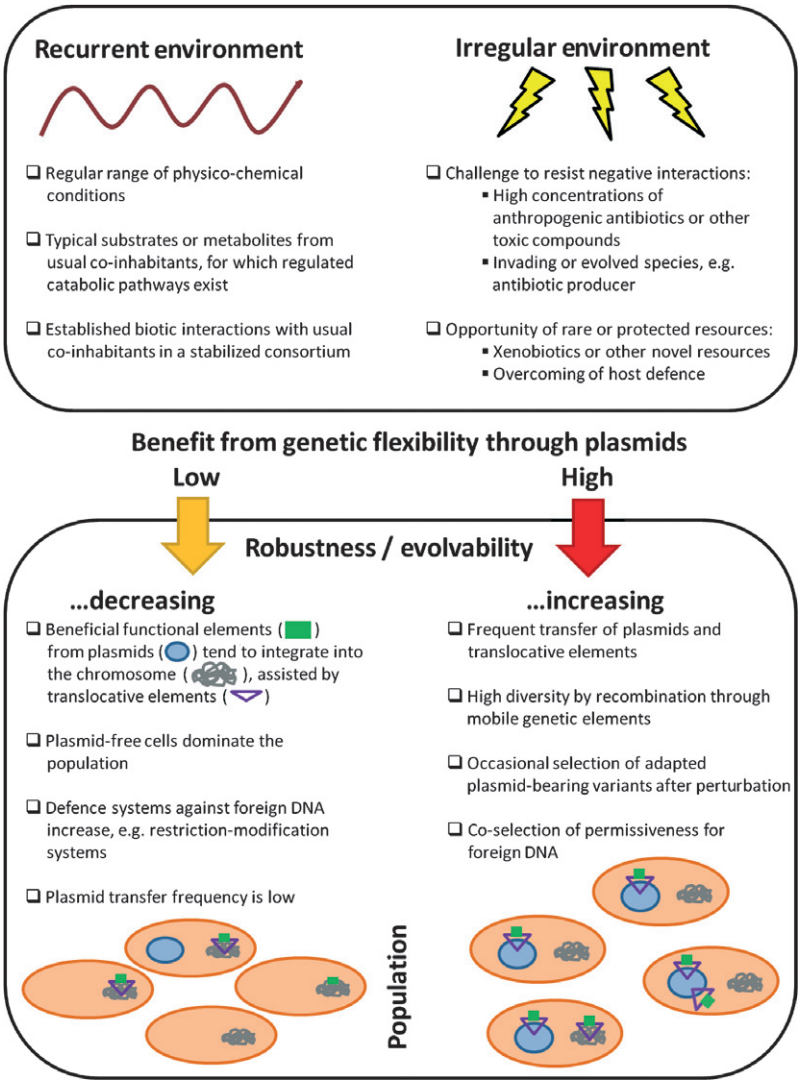

Fig. 5. Contribution of broad-host-range plasmids to the adaptability of bacterial populations in soil when the environment shows a constant or long-term recurring range of conditions vs. an irregular challenge or opportunity. Adapted by L. Bandounas.

for adaptation to environmental challenges than genetic innovation by mutations (Jain *et al.*, 2003; Hermisson & Pennings, 2005). Thus, the primary benefit of bacterial populations from maintaining transferable plasmids might be that they gain robustness in the uncertainty of irregular environments (Fig. 5).

An obvious example of environmental uncertainty is the challenge of antibiotic compounds resulting in the enrichment of those subpopulations that have acquired antibiotic resistance genes. Other examples of environmental uncertainty are the flexible immune systems of plant or animal hosts, which can be undermined by bacterial pathogens through acquisition of genomic islands (Hochhut *et al.*, 2006; Arnold *et al.*, 2007), or the occurrence of recalcitrant compounds, which can be utilized through catabolic pathway assembly (Top & Springael, 2003). In these cases the environment provides an irregular opportunity to access rare or protected resources.

More generally, simulations have shown that the net growth rate of heterogeneous populations may be larger than of homogeneous populations in fluctuating environments (Thattai & van Oudenaarden, 2004; Gander *et al.*, 2007). A secondary benefit from permissiveness to plasmids may be that genetic variants are more frequently generated that have the potential to become adaptive in the course of evolution. This heritable quantitative trait is often referred to as evolvability (Lenski *et al.*, 2006).

The frequency of irregular events in the environment of a bacterial soil population may change over time. How

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can the population balance the risk of acquiring deleterious genes with the benefits derived from genetic flexibility by plasmid acquisition? This can be achieved if the bacterial population is heterogeneous in its permissiveness to foreign DNA. A well known example is *Bacillus subtilis*, where only a minority of the population becomes competent in DNA uptake (Dubnau & Losick, 2006). For the same reason, but by different mechanisms, plasmid uptake might vary between cells of a population. Strains of *P. putida* can differ largely in their ability to host IncP-1 plasmids (Heuer *et al.*, 2007), suggesting a heterogeneous distribution of protective mechanisms.

Only one study so far has investigated heterogeneity within a local population with respect to the capacity to receive BHR plasmids (Heuer *et al.*, 2010). Interestingly, the permissiveness for BHR IncP-1 plasmids varied by orders of magnitude among genetically indistinguishable strains of *Dickeya* sp., which were isolated from the rhizosphere of potato plants within an area of 400 m^2. The strain-specific permissiveness was reproducible under different mating conditions and was shown for both the IncP-1α plasmid pTH10 and the IncP-1β plasmid pB10. This supports a model, where the cost of foreign DNA and the risk of acquiring deleterious genes is taken over by permissive individuals, while the population as a whole benefits from genetic flexibility (Jain *et al.*, 2003; Hermisson & Pennings, 2005; Heuer *et al.*, 2008). Adaptation of a bacterial population to more frequent irregular challenges or opportunities will occur as a consequence of occasional fitness advantages of permissive individuals, which are more likely to acquire genes to respond to the perturbation (Fig. 5). Their relative enrichment increases the net flexibility of the population. An implication of this is that our efforts to control bacterial pathogens might be undermined because, by the same means that challenge the population, we select for strains with higher adaptive potential. Strains with a higher permissiveness for MGEs will decrease the time until resistance to a newly developed antibiotic compound is widespread.

Conclusion

Over the last decade, sequencing of plasmids or complete genomes of soil bacteria has provided important insights into the plasmid-mediated plasticity of soil bacteria. Despite a metabolic burden that is assumed to be connected with plasmid carriage, plasmids appear to be an ancient and successful strategy to ensure survival of a population in spatial and temporal heterogeneous conditions with various environmental stresses or opportunities that occur irregularly or as a novel challenge. MGEs are assumed to cause micro-diverse patchy populations in soil, which are more likely to include adaptive variants. The spatial structure and heterogeneity in soil is assumed to stabilize this micro-diversity. The generation and maintenance of genetic variation in particular by BHR plasmids in order to gain robustness to irregular environmental conditions has been proposed as a general ecological strategy of bacterial populations (Heuer *et al.*, 2008).

Comparative genomics with increasing numbers of available plasmid sequences revealed that the accessory gene load of different plasmid groups is a major reason for the enormous diversity of plasmids observed. The increasing numbers of plasmid sequences are also the prerequisite of improved molecular detection systems that can now be employed to explore the diversity of different plasmid groups in different environmental habitats and to quantify plasmid abundance in correlation with different environmental factors. Furthermore, recent methodological advances enable the study of gene expression and interaction with the plasmid host. However, whereas real *in situ* transconjugants in soil have been reported in studies mainly working with inoculated plasmid donors or recipients, the detection of transfer events between indigenous plasmid donors and recipients remains challenging. Most of the studies on plasmid transfer in bulk and rhizosphere soils were done in the 1990s with cultivation-based methods. In spite of the important role of plasmid-mediated bacterial adaptation and diversification, our understanding of plasmid persistence and transfer in spatially structured environments is still poor (Fox *et al.*, 2008) and there remain many methodological challenges for studying HGT at a single cell level in soil. Recent tool developments will hopefully also open the black box soil and shed more light on factors, e.g. the sub-inhibitory concentration of antibiotics in soil triggering HGT events. DNA-based quantitative data on plasmid abundance, single cell analysis by flow cytometry as well as transcriptomic and proteomic tools will be instrumental in testing theories developed with fast growing broth cultures under soil conditions.

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