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# Role of Plasmids in Plant-Bacteria Interactions

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**Abstract**
Plants are colonized by diverse microorganisms, which may positively or negatively influence the plant fitness. The positive impact includes nutrient acquisition-enhancement of resistance to biotic and abiotic stresses, both important factors for plant growth and survival, while plant pathogenic bacteria can cause diseases. Plant pathogens are adapted to negate or evade plant defense mechanisms, e.g. by the injection of effector proteins into the host cells or by avoiding the recognition by the host. Plasmids play an important role in the rapid bacterial adaptation to stresses and changing environmental conditions. In the plant environment, plasmids can further provide a selective advantage for the host bacteria, e.g. by carrying genes encoding metabolic pathways, metal and antibiotic resistances, or pathogenicity-related genes. However, we are only beginning to understand the role of mobile genetic elements and horizontal gene transfer for plant-associated bacteria. In this review, we aim to provide a short update on what is known about plasmids and horizontal gene transfer of plant-associated bacteria and their role in plant-bacteria interactions. Furthermore, we discuss tools available to study the plant-associated mobilome, its transferability, and its bacterial hosts.

**Introduction**
Plant-associated microorganisms are considered to be of great importance for plant health, plant productivity and ecosystem functioning. They expand the metabolic repertoire of plants, increase the resource uptake and provide novel nutritional and defense pathways (Berendsen et al., 2012; Berg et al., 2014). Therefore, the genetic information provided by the plant microbiome is also called the second genome of the plant (Berendsen et al., 2012). In the phytosphere, mutualistic associations were studied in great detail for rhizobia and mycorrhizae, rhizobacteria with plant growth promoting or biocontrol activity. However, also parasitic interactions with plant pathogens are well-studied today. Plants are able to influence soil properties, e.g. by the release of nutrients and secondary metabolites via root exudation, which are used to combat pathogenic microorganisms while attracting beneficial ones (Badri et al., 2009; Philippot et al., 2013). At the same time, these rhizodeposits (nutrients, exudates, border cells and mucilage) released by the plants to the rhizosphere (soil influenced by the root) are thriving soil microbial growth, density and activity, which are prerequisites for horizontal gene transfer (HGT) (Kroer et al., 1998; Mølbak et al., 2007; Philippot et al., 2013; Pukall et al., 1996; Raaijmakers et al., 2009; van Elsas et al., 2003). The molecular characterization of strains often revealed that the presence of plasmid-encoded products plays a role in the interaction with the plant. Not only the rhizosphere, also the phyllosphere of plants is considered to be conducive to HGT, which can (positively) affect host fitness (van Elsas et al., 2003). Recently, the development and application of tools such as next generation sequencing contributed to understand the role of mobile genetic elements (MGEs) and HGT in the structure, function and evolution of plant-associated bacterial

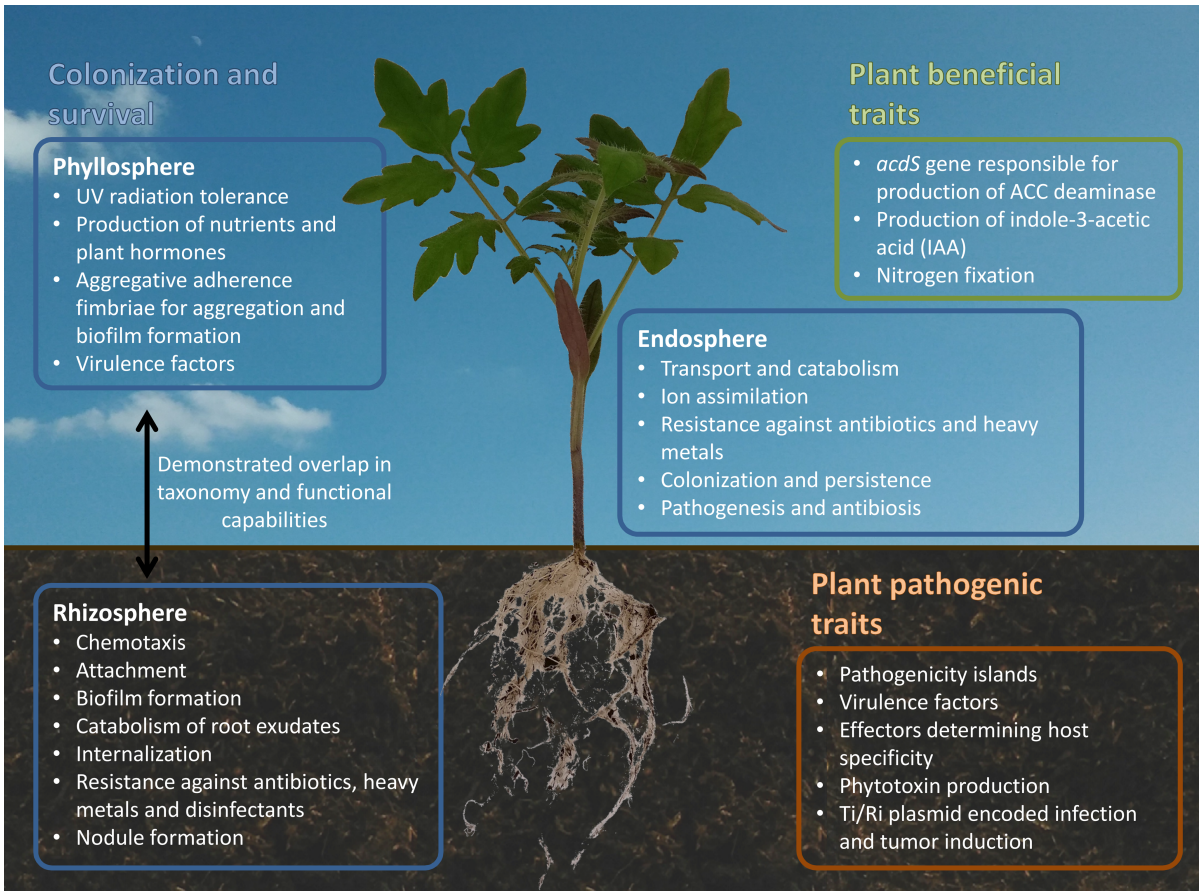



**Figure 1.** Plasmid-encoded functions in the phytosphere that are described so far. Plasmid-encoded functions are sorted by colonization and survival (blue boxes), plant beneficial (green box) and plant pathogenic traits (brown box). Plant surfaces are considered as hot spots of bacterial conjugation, and an overlap in taxonomy and functional capabilities was already demonstrated between phyllosphere and rhizosphere bacterial communities.

communities in the phytosphere. In this review, we aim to give a short update on plasmids in plant-associated bacteria, HGT and their role in plant-bacteria interactions with a special focus on rhizosphere, phyllosphere and endosphere (summarized in Figure 1). Furthermore, recent methodological developments will be discussed regarding their potential to investigate the plant-associated mobilome and the respective bacterial hosts.

## Horizontal gene transfer (HGT) in the phytosphere

HGT has a strong influence on the bacterial evolution (Jain et al., 2002; Koonin et al., 2001; Koski et al., 2001; Ochman et al., 2000; van Elsas et al., 2003). Plasmids belong to the most important vectors for HGT. They provide an efficient mean for rapid bacterial adaption to changing environmental conditions. Additionally to the core (backbone) genes that include plasmid replication, maintenance and transfer, plasmids typically carry a flexible (accessory) gene pool (Heuer and Smalla, 2012). Flexible genes carried by plasmids are known to code for detoxification, virulence, ecological interactions and antibiotic resistance (Smillie et al., 2010), but can also include catabolic pathways (Dennis, 2005). Recombination with the host chromosome and with other plasmids can lead to an acquisition or loss of these functions, resulting in a mosaic and modular genetic composition (Norman et al., 2009; Toussaint and Merlin, 2002). It was reported that a high proportion of bacteria isolated from the phytosphere carry plasmids that are

characterized by a high diversity in terms of function and genetic relatedness (Viegas et al., 1997). Furthermore, the phytosphere is constituted by habitats differing with respect to environmental conditions and surface characteristics that require specific adaptations of the colonizing bacteria and provide diverse conditions for HGT. Therefore, in the following chapters, plasmids in bacteria colonizing the different plant habitats, namely rhizosphere, phyllosphere and endosphere are separately discussed in terms of presence, transferability and potential function.

**Plant-bacteria interactions in the rhizosphere and the role of plasmids**

*Root exudates are shaping the rhizosphere bacterial community*

The rhizosphere is defined as the soil that is surrounding the root and influenced by the plant (Hartmann et al., 2008a; Hiltner, 1904). In this environment, the plant is in tight contact with soil-borne microorganisms, which include beneficial, saprophytic and pathogenic bacteria, all having a great impact on plant growth and health (Berendsen et al., 2012; Hayat et al., 2010). Plants are able to shape the rhizosphere bacterial community by releasing a wide range of so-called root exudates, which include mono- and polysaccharides, amino acids, sterols, phenols, enzymes, plant growth regulators and different other secondary metabolites (Bais et al., 2006). These compounds form gradients in soil, which attract motile bacteria chemotactically and select for a specific bacterial community (Badri et al., 2009; Bais et al., 2006; Philippot et al., 2013). Accordingly, it was shown for soil bacteria that most genomes contain chemotaxis and mobility genes, providing a competitive advantage in the colonization of plant root surfaces and leading to an enrichment of bacteria carrying chemotaxis-encoding genes in the rhizosphere compared to bulk soil (Scharf et al., 2016). These genes associated with motility but also with adhesion and biofilm formation can be located on plasmids, as described, for example, for pCSA2 of *Cronobacter sakazakii* (Choi et al., 2015). Another indication for the importance of plasmids regarding the rhizosphere competence of bacteria was observed for the plant beneficial bacterium *Bacillus amyloliquefaciens* subsp. *plantarum* S499 that carries a plasmid with seven genes coding for traits predicted to be important for the colonization of plant roots (Molinatto et al., 2016).

In the rhizosphere, the abundance of micro-organisms is higher than in the surrounding bulk soil. However, the evenness is decreased, likely because of the higher availability of carbon and other nutrients released by plant roots (Hartmann et al., 2008b; Kandeler et al., 2002). For example, a considerable amount of the carbon produced by photosynthesis is released by the roots (Marschner, 1995). The microbes able to utilize these nutrients can proliferate. Therefore, the acquisition and exchange of additional metabolic pathways by HGT might be a successful strategy. For example, the self-transmissible plasmid pRme41a, which has been isolated from *Ensifer meliloti* 41, codes for catabolism of root exudates which might be important for the competitiveness in the rhizosphere (Tepfer et al., 1988). Wang et al. (2007) isolated bacteria from the rhizosphere of *Zea mays,* which carried several plasmids conferring the ability to degrade phenol and depicted the connection to polluted sites. Lilley and Bailey (1997) linked fitness advantage of bacteria in the rhizosphere to the acquisition and carriage of plasmids. Furthermore, the cryptic gene-mobilizing plasmid pIPO2 isolated from wheat rhizosphere was found to be a highly proficient IncQ plasmid mobilizer (van Elsas et al., 1998). This plasmid was the first isolated from the PromA group, which is a typical plasmid group of the rhizosphere (Van der Auwera et al., 2009). The whole plasmid was sequenced and its prevalence in soil was assessed (Tauch et al., 2002).

In another study, Jechalke et al. (2014b) observed an enrichment of *korB* genes specific for IncP-1 plasmids in total community (TC)-DNA extracted from the rhizosphere of lettuce compared to bulk soil. However, the isolation and characterization of IncP-1 plasmids from the rhizosphere would be required to gain insights into the traits conferred. In conclusion, plasmids encoding chemotaxis, mobility, colonization and metabolic pathway-associated traits might provide a fitness advantage to their bacterial hosts colonizing the rhizosphere leading to proliferation of plasmid-containing bacteria. Higher plasmid transfer frequencies in the rhizosphere compared to bulk soil are assumed to be a result of the increased abundance of plasmid donors and also of their increased metabolic activity (Pukall et al., 1996).

*Soil pollutants*

The rhizosphere is a highly dynamic and heterogenous habitat that is shaped by many biotic and abiotic factors. Among these factors are man-made pollutants such as antibiotics, heavy metals,

disinfectants and pesticides that can enter the soil e.g. via sewage sludge, digestates, manure or other organic fertilizers (Heuer et al., 2012; Schlüter et al., 2007; Slater et al., 2008; Top et al., 1995; Wolters et al., 2016), and are assumed to increase the abundance of plasmid carrying bacteria. Soil and plant-associated bacteria can adapt to, and cope with these pollutants by means of genes frequently located on MGEs such as plasmids. Bacteria carrying plasmids might be indigenous soil bacteria or introduced to soil, e.g. with organic fertilizers (Binh et al., 2008; Dröge et al., 1999; Hall et al., 2015; Heuer et al., 2012; Jechalke et al., 2014a; Lilley and Bailey, 1997; Malik et al., 2008). Plasmid-mediated traits can provide a fitness advantage for their hosts in case of selective pressure. This was demonstrated for a sulfonamide resistance-carrying LowGC-type plasmid in *Acinetobacter baylyi* BD413 applied to soil treated with manure spiked or unspiked with the veterinary antibiotic sulfadiazine (Jechalke et al., 2013b). Narrow host range (NHR) plasmids, such as the LowGC plasmids, were frequently captured by exogenous isolation from manured soil, maize or grass rhizospheres (Kopmann et al., 2013) and *Acinetobacter* spp. were identified as potential hosts, suggesting their importance for human medicine (Heuer et al., 2009).

Besides antibiotic resistance, plasmids can shuttle other useful traits like heavy metal resistance genes, efflux pumps and toxin-antitoxin systems (reviewed by Heuer and Smalla (2012)). These might provide fitness advantages for their host in the rhizosphere. Accordingly, correlations between the abundance of plasmids and pollution in soils were proposed in numerous studies (de Lipthay et al., 2008; Dealtry et al., 2014; Gstalder et al., 2003; Heuer et al., 2009; Heuer and Smalla, 2012; Smalla et al., 2006; Top et al., 1995), supporting the hypothesis that plasmids are very important for bacteria to cope with, or benefit from soil pollutants in the rhizosphere.

**Phyllosphere bacterial communities and their plasmids**

The phyllosphere is defined as the aerial surface of the plants. Therefore, tolerance to UV exposure, nutrient and water limitations, as well as high temperature shifts and the presence of reactive oxygen species (ROS) are properties required to successfully colonize the phyllosphere (Knief et al., 2011; Lindow and Brandl, 2003; Newton et al., 2010). Phyllosphere-associated bacteria are supposed to play an important role in plant host growth, fitness and productivity, e.g. by production of growth-promoting nutrients and hormones, protection against pathogen infection, and indirectly influence plant population dynamics and community diversity (Kembel et al., 2014; Laforest-Lapointe et al., 2016; Laforest-Lapointe et al., 2017). Nutrients provided by the leaf can attract bacteria by chemotaxis, as already discussed for the rhizosphere. Furthermore, it was demonstrated that light-treatment of iceberg lettuce leaves resulted in aggregation of *Salmonella enterica* serovar Typhimurium near open stomata and invasion into the inner leaf tissue (Kroupitski et al., 2009). Attachment of bacteria to plant cells is considered to be the next step required in plant bacteria interactions and the formation of biofilms (Rodríguez-Navarro et al., 2007), which can be also associated with MGEs. For example, plasmid-encoded aggregative adherence fimbriae I (AAF/I) of *E. coli* O104:H4 were shown to play a crucial role in aggregation and biofilm formation on spinach and abiotic surfaces (Nagy et al., 2016). Genes linked to type IV secretion systems and extracellular polymeric substance (EPS) synthesis were also detected by Schmeisser et al. (2009) on the megaplasmid pNGR234*b* of *Rhizobium* sp. strain NGR234. Members of the pPT23A-family were reported to likely contribute to virulence and ecological fitness in *Pseudomonas syringae* pathosystems, e.g. by encoding methyl-accepting chemotaxis proteins or conferring UV radiation tolerance (Cazorla et al., 2008; Sundin et al., 2004). Furthermore, bacteria of the genus *Pseudomonas* are frequently found in association with plants and can have significant impact on agriculture by acting as mutualists, saprophytes or pathogens (Espinosa-Urgel, 2004).

Plant surfaces in general are regarded as hotspots of bacterial conjugation events (van Elsas et al., 2003). Considering that the estimated global leaf area is larger than the plain surface of the planet and colonized on average by $10^6$-$10^7$ bacterial cells/ $cm^2$ (Lindow and Brandl, 2003; Ortega et al., 2016; Woodward and Lomas, 2004), the phyllosphere has an enormous potential for bacterial evolution and adaptation by HGT processes. However, the knowledge of plasmid transfer in and on plants is still scarce compared to other environments. Several reports documented high rates of plasmid transfer in the phyllosphere. For example, Normander et al. (1998) examined the transfer of TOL plasmids on the phytoplane of bean (*Phaseolus vulgaris*) and found the plasmid from the donor *Pseudomonas putida* KT2442 in 33% of *P. putida* KT2440 recipients. The transfer ratios on

leaves were reported to be 30 times higher than on polycarbonate filters (Normander et al., 1998). Conjugal plasmid transfer between two *P. putida* strains on the phylloplane of bean was positively related to the spatial distribution of the bacteria into microhabitats such as junctures between epidermal cells and substomatal cavities (Normander et al., 1998). Furthermore, the authors concluded that leaf exudates ensured that the activity of the bacteria was sufficient to allow conjugative transfer.

In another study, mercury resistance plasmids were acquired by conjugation from natural bacterial communities of sugar beet roots and leaves to a *Pseudomonas fluorescens* recipient, previously isolated from sugar beets (Lilley and Bailey, 1997), demonstrating that gene transfer within the plant surface compartments might be a common event.

**Plasmid-mediated resistome associated with produce**

The high potential for HGT in the phyllosphere plays an important role in dissemination of antibiotic resistance genes (ARGs). Conjugal transfer of plasmid-mediated antibiotic resistance from pseudomonads to other *Pseudomonas* spp., Enterobacteriaceae members and to diverse indigenous bacteria occurs on growing alfalfa sprouts (Mølbak *et al.*, 2003) and on the leaves of bean plants (Björklöf et al., 1995). However, produce can be a source of *Escherichia coli* and other Enterobacteriaceae carrying a diverse set of ARGs located on MGEs (Ben Said et al., 2015; Jones-Dias et al., 2016). Conjugative plasmids isolated from produce were shown to carry resistances against antibiotics and against heavy metal compounds or disinfectants, making co-selection possible (Ben Said et al., 2015; Kim et al., 2015; Verraes et al., 2013). Previous studies reported that Enterobacteriaceae and *Pseudomonas* spp., isolated from raw vegetables harbored NHR plasmid groups like IncF, IncI, IncY, and IncX and broad-host-range (BHR) plasmid groups such as IncP, IncH, IncQ IncM/L. These replicon types can carry genes conferring resistance to different antibiotic classes including aminoglycosides, beta-lactams, phenicols, tetracyclines, sulfonamides, and quinolones (Araújo et al., 2017; Ben Said et al., 2015; Jones-Dias et al., 2016; Rahube et al., 2014; Veldman et al., 2014). In addition, the IncF plasmids can also carry virulence genes (Johnson and Nolan, 2009; Villa et al., 2010). Recently, Enterobacteriaceae were isolated from vegetables, soil and irrigation water, which harbored IncI1, IncF, IncK, IncY, and IncB/O plasmids that were reported to carry extended-spectrum beta-lactamase (ESBL) genes (*bla* $_{CTX-M}$) (Ben Said et al., 2015; Kim et al., 2015; Njage and Buys, 2015; Reuland et al., 2014). Mellmann et al. (2011) reported that the NHR IncI1 plasmids carrying *bla*$_{CTX-M-15}$ and *bla*$_{TEM-1}$ have been associated with an outbreak of *E. coli* O104 in Germany in 2011. Interestingly, *E. coli* and *Raoultella ornithinolytica* strains recovered from lettuce were found to carry IncHI2, IncI2, and IncX4 plasmids coding for colistin resistance (*mcr-1*) (Luo et al., 2017). Furthermore, plasmid-mediated quinolone resistance (PMQR) genes (*qnrB*, *qnrS*, and *aac(6')-1b-cr*) have been reported in Enterobacteriaceae isolated from fresh herbs, located on IncHI1, IncHI2, IncR, and IncF plasmids (Veldman et al., 2014). Thus, the NHR and BHR plasmids have the potential to be major contributors to the proliferation of ARGs and could pose a potential risk to human health by consumption of produce. However, these studies neglected HGT processes in the plant endosphere, those will be discussed in the following paragraph.

**Bacterial plant endophytes**

Endophytes are defined as organisms that live or at least persist inside of plants without visibly harming them (Hallmann et al., 1997; Hardoim et al., 2015). Recent technological advances in the field of microbiology and "omics" methods provided fascinating insights into the functional characteristics of endophytes and endophytic communities. In many cases, the coexistence of plant and bacteria had a positive influence on plant growth and the tolerance towards different stresses (Compant et al., 2010; Miliute et al., 2015; Santoyo et al., 2016). The internal root microbiome is typically made up of a community of endophytes interacting and competing with each other (Gaiero et al., 2013). Although not much is known so far about the role of plasmids in endophyte bacterial communities, plasmids might help endophytes to successfully survive, compete within the endophyte community and persist within the plant. In this respect, a cryptic plasmid from an endophytic *Pantoea agglomerans* was isolated from eucalyptus and four potential ORFs were identified with unknown function (de Lima Procópio et al., 2011). In another work, large *Pantoea* plasmids (LPP-1) of 20 *Pantoea* strains including pathogens and endo-/epiphytes were compared, it was revealed that these plasmids contribute to important cell functions like thiamine biosynthesis and played a major role in the adaptation to their ecological niches and functional specialization due to variable elements with genes for the transport and catabolism of diverse metabolic substrates (De Maayer et al., 2012). The small cryptic plasmid pLK39 isolated

from a leaf endophytic *Salmonella* sp. carried putative mobilization genes and a replication control region with a high similarity to plasmids isolated from plant pathogenic *Erwinia* species and further ORFs with unknown functions (Dourado et al., 2014), further indicating a potential importance of plasmids for plant colonization. HGT among plant-associated endophytic bacteria might be used by the endophytic bacterial community to adapt to changing environmental conditions. This was demonstrated by Taghavi et al. (2005), who observed the transfer of a toluene degradation pathway located on plasmid pTOM-Bu61 from *Burkholderia cepacia* strains to members of the indigenous plant bacterial communities of poplar. These examples indicate that HGT processes within endophyte bacterial communities are of importance for bacterial adaptation and survival within the host plant.

**Plasmids of plant beneficial bacteria**

Probably one of the best investigated examples of beneficial plant-bacteria relationships is the symbiosis of leguminous plants with bacteria belonging to the genus *Rhizobium*. The nodule inducing rhizobia are attracted by flavonoids released by the plant resulting in the activation of the plasmid-encoded *nod* operon (Downie, 2010; Venturi and Keel, 2016). The specific expression of genes in both partners results in cellular differentiation processes leading to nodule development and bacterial invasion, finally allowing the bacteria to fix atmospheric nitrogen that can be utilized by the plant (D'Haeze and Holsters, 2002). Nodulation genes of rhizobia, which are involved in the production of Nod factors are primarily located on large symbiotic plasmids, also referred to as Sym plasmids or pSyms (Bánfalvi et al., 1981; Rosenberg et al., 1981). In rhizobia, a distinction can be made between two types of plasmids. Quorum sensing (QS)-regulated plasmids can be transferred by conjugation when the cell density is high enough. Genes encoding the QS regulatory system can be found on the rhizobial plasmids pRL1jI, pRleVF39, pRetCFN42 and pSmed (Ding and Hynes, 2009). Furthermore, some plasmids code for the genes *rctA/B.* While RctA suppresses the transcription of *virB* by binding to the promoter region, RctB is able to antagonize the effect. The *rctA/B* genes can be found on the plasmids pAtC, pRetCFN, pretCIAT and pSymA (Ding and Hynes, 2009). The different plasmid transfer systems of rhizobia were reviewed by Ding and Hynes (2009) suggesting that plasmids play an important role in rhizobia.

In the plant growth-promoting rhizobacterium *Azospirillum brasilense,* plasmids were suggested to mediate bacteria-root-interactions by carrying genes encoding lipopolysaccharides (LPS), EPS and polar and lateral flagella (Fibach-Paldi et al., 2012; Vanbleu et al., 2004). Many plant beneficial soil bacteria were found to produce plant hormones and some bacteria have 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase genes (Dimkpa et al., 2009; Glick, 2012). For example, *Pseudomonas fluorescens* FY32 was reported to carry a plasmid of 50 kb which codes for the ACC-deaminase gene *acdS* (Farajzadeh et al., 2010). Phylogenetic analysis of ACC-deaminase genes indicated HGT potential of this plasmid (Hontzeas et al., 2005). Genes encoding ACC-deaminase were reported to occur on the chromosome but also on plasmids (Nascimento et al., 2014). ACC-deaminase is able to reduce the ethylene level in plants under stress conditions by cleaving ACC, the immediate precursor of ethylene (Gontia-Mishra et al., 2014). The plant phytohormone auxin plays also a role in the plant's defense system against phytopathogenic bacteria (Spaepen and Vanderleyden, 2011). One of the major naturally occurring hormones of the auxin class, Indole-3-acetic acid (IAA) was found to be plasmid-encoded in wheat root associated *Acinetobacter* strains (Huddedar et al., 2002). This plant hormone is known to affect plant growth and immune response, and it can be produced and used by bacteria as a signaling molecule (Spaepen et al., 2007). Bacteria can use IAA to circumvent the plant defense by de-repressing the auxin signaling in the plant. IAA can also be used as a signaling molecule by bacteria, inducing the expression of genes related to survival under stress conditions (Remans et al., 2006). Localization of genes on high copy plasmids can be used by bacteria to upregulate the expression of genes that are important for plant-bacteria interactions. For example, the localization of auxin biosynthesis genes on multicopy plasmids can lead to a higher IAA production (Spaepen and Vanderleyden, 2011).

Besides hormones there are different other mechanisms of protecting the plant like the secretion of EPS and the induction of plant heat shock proteins and osmo-protectants (Grover et al., 2011). Many EPS-related genes were found to be localized on plasmids in different bacteria (Schmid et al., 2015). Plasmid carriage of EPS-related genes might be advantageous due to the copy number effects.

**Plasmids of plant pathogenic bacteria**

Plant pathogenic bacteria are responsible for extensive losses in agriculture. Plasmids are generally widely distributed in plant pathogenic bacteria and may carry traits relevant for virulence and ecological fitness of its hosts. Also the host specificity of the pathogen *P. syringae* is determined by effectors that are encoded on plasmids (Bever et al., 2012). A large native plasmid detected in the bean pathogen *P. syringae* pv. *phaseolicola* was found to carry genes encoding type III effectors and other potential virulence genes (Jackson et al., 1999). Furthermore, production of chlorosis-inducing phytotoxin coronatine and an ethylene-forming enzyme, were found to be encoded by plasmids in some pathovars (Alarcón-Chaidez et al., 1999; Bender et al., 1991; Weingart et al., 1999). For the plant pathogenic bacterium *Xanthomonas campestris* pv. *campestris*, it was suggested that the trans-acting sRNA Xcc1 was originally captured by integrons from natural environments and then spread among bacterial species by HGT, mediated by transposons and plasmids (Chen et al., 2011). *X. campestris* can cause black rot disease in cruciferous crops, but it can also be found in asymptomatic association with plant tissue, or as epiphyte (Maas et al., 1985). Marques et al. (2001) analyzed the genome of the plant pathogenic bacterium *Xylella fastidiosa* and revealed two plasmids. In the analysis of the genes present on the plasmids, some ORFs were found that might be important for virulence, and one ORF showed similarity to the virulence-associated protein VapD. *Erwinia* spp. plasmids were found to carry traits such as streptomycin resistance genes, but also pathogenicity and virulence factors such as EPS production and type III secretion systems (T3SSs), which might have caused the emergence of pathogenic from the non-pathogenic species by HGT (Llop, 2015). Furthermore, *Pseudomonas savastanoi*, the causal agent of olive knot disease, harbors plasmids that affect the virulence and plant host range (Bardaji et al., 2011; Eltlbany et al., 2012; Pérez-Martínez et al., 2008). Pathogenicity of *Pantoea agglomerans* causing tumors on various plants is mainly encoded by a plasmid designated as pPATH (Manulis and Barash, 2003). This plasmid carries various virulence genes, including *hrp* cluster and genes encoding type III virulence effectors and biosynthesis of phytohormones. It was also demonstrated that pathogenicity of most *Rhodococcus* spp., causal agents of leafy gall disease on a wide range of host plants, may be associated with a linear plasmid (Creason et al., 2014).

In terms of pathogenesis, tumorigenic and rhizogenic *Rhizobiaceae* species (here collectively called agrobacteria) are certainly the most extensively studied group of phytopathogenic bacteria interacting with plants in the rhizosphere through plasmid-encoded functions. Tumorigenic and rhizogenic Rhizobiaceae strains causing crown gall and hairy root diseases, respectively, are predominantly soil-inhabiting bacteria that are able to colonize rhizosphere and different plant tissues (Bosmans et al., 2017; Burr et al., 1998; Burr and Otten, 1999; Escobar and Dandekar, 2003; Kuzmanović et al., 2018; Otten et al., 2008; Puławska, 2010). They may also systemically colonize host plants and can be present in asymptomatic plant tissue (Otten et al., 2008). Numerous plants can be infected by these bacteria, including important agricultural crops such as fruit species, grapevine and perennial ornamentals. Symptoms of crown gall include tumor formation on roots, crowns, trunks and canes of infected plants. On the other hand, hairy root disease is characterized by abnormal rooting of the host plant. Naturally occurring pathogenic strains are generally distributed within the genus *Agrobacterium*, and species *Allorhizobium vitis* and *Rhizobium rhizogenes*. In the next section, the role of plasmids in plant-bacteria interaction and pathogenesis of bacteria associated with crown gall and hairy root diseases will be discussed in more detail.

*Plasmids in bacteria associated with crown gall and hairy root diseases*

Bacteria associated with crown gall and hairy root diseases may carry a variable number of plasmids. However, their ability to cause neoplastic diseases is mainly encoded by tumor-inducing (Ti) and root-inducing (Ri) plasmids, carried by tumorigenic and rhizogenic strains, respectively. In addition, plasmids can highly determine the host range of agrobacteria, as shown for the Ti plasmid (Knauf et al., 1982; Thomashow et al., 1980). It was shown that *Agrobacterium fabrum* C58 strains lacking the Ti plasmid did not bind to plant protoplasts, which is likely associated with the virulence induced type IV secretion systems (T4SSs) (Aguilar et al., 2011; Matthysse et al., 1978). The size of Ti/Ri plasmids can range from around 170 kbp to more than 250 kbp. The Ti and Ri plasmids are genetically related and belong to the *repABC* family of megaplasmids (Christie and Gordon, 2014; Pappas and Cevallos, 2011; Suzuki et al., 2009). The Ti/Ri plasmids consists of several functional elements including: transferred DNA (T-DNA), virulence (*vir*) region,

opine utilization genes, replication (*rep*) region, and conjugative transfer genes (*tra* and *trb* loci). T-DNA contains genes that are expressed in the host plant and they may be divided into two groups: oncogenes that direct synthesis of phytohormones (Britton et al., 2008), and gene clusters involved in synthesis of special classes of compounds, typically conjugates of amino acids and α-ketoacids or sugars, called opines (Chilton et al., 2001; Dessaux et al., 1998).

Wounds caused by abiotic or biotic factors serve as an entry points for agrobacteria and are considered to be necessary for the initiation of infection, which primarily occurs in the rhizosphere. Interestingly, transformation of unwounded plants by agrobacteria has also been demonstrated (Brencic et al., 2005). Although transformed plants produced opines, tumor formation was absent. The infection itself is a complex process of interkingdom DNA transfer and represents an example of natural genetic transformation of plants. Mechanisms of pathogenesis induced by agrobacteria, including the history of this research field, have been comprehensively reviewed (Gelvin, 2012; Hooykaas, 2000; Kado, 2014; McCullen and Binns, 2006; Nester, 2014; Pitzschke and Hirt, 2010; Zhu et al., 2000; Zupan et al., 2000).

Opines produced by transformed plants, mostly in tumors or hairy roots, serve as a selective nutrient source for the disease-causing rhizobia. The presence of opines is not limited to tumor tissue, they can be translocated to the other parts of the plant and excreted from their roots. Therefore, they may affect also the composition of the bacterial community in the rhizosphere (Mansouri et al., 2002; Oger et al., 1997; Savka et al., 1996; Savka and Farrand, 1997). Moreover, some opines induce the conjugative transfer of Ti plasmids, which is regulated by the QS system belonging to the LuxR/LuxI class (Dessaux et al., 1998; Farrand, 1998; Lang and Faure, 2014; White and Winans, 2007). Agrobacteria may carry some other plasmids that are associated with plant-microbe interactions, which can also specifically interact with Ti/Ri plasmids (Otten et al., 2008; Platt et al., 2014). Thus, non-pathogenic and pathogenic Rhizobiaceae strains isolated from tumors or soil around diseased plants may carry opine-catabolic (OC) plasmids, which contain genes encoding uptake and catabolism of opines as Ti/Ri plasmids (Merlo and Nester, 1977; Petit et al., 1983; Puławska et al., 2016; Szegedi et al., 1999; Wabiko et al., 1990; Wetzel et al., 2014). Unlike Ti/Ri plasmids, OC plasmids do not carry *vir* genes and T-DNA. However, non-pathogenic strains harboring OC plasmids could benefit from opines produced in tumors and in hairy roots. *A. vitis* associated with grapevine crown gall may harbor another type of plasmids, responsible for utilization of tartrate. These plasmids most likely enhance competitiveness of this pathogen on grapevine, since tartrate is an abundant compound in this plant species (Kado, 1998; Salomone et al., 1998; Szegedi et al., 1992). The well-known biological control strain *R. rhizogenes* K84 shows antimicrobial activity against a certain spectrum of agrobacteria, and it was shown to carry the plasmids pAgK84 and pAtK84a that are associated with the synthesis of the specific antibiotics agrocin 84 and 434, respectively (Donner et al., 1993; Ellis et al., 1979; Kim et al., 2006; McClure et al., 1998). Additionally, this strain carries an OC plasmid (pAtK84b) conferring competitiveness of this strain within tumors. Moreover, studies on interaction between Ti plasmid (pTiC58) and accessory megaplasmid (pAtC58) coexisting in *Agrobacterium* sp. C58, suggested that pAtC58 may have a positive effect on tumor size (Nair et al., 2003) and that different forms of this plasmid which arose by deletions can affect *vir* gene expression in pTiC58 (Morton et al., 2013). It was also demonstrated that this plasmid provides bacteria with competitive advantage in the rhizosphere (Morton et al., 2014). Interestingly, some strains of *Agrobacterium* spp. and *R. rhizogenes* may harbor symbiotic (Sym) plasmids and effectively colonize legume plants (Cummings et al., 2009; Velázquez et al., 2005; Zhao et al., 2014).

## Tools to investigate plasmids and horizontal gene transfer in the phytosphere

### *Cultivation-dependent and -independent methods to investigate plasmids*

Methods available for plasmid detection and characterization were recently reviewed by Smalla et al. (2015). The vast majority of studies on plasmids of phytosphere-associated bacteria is still based on isolates and thus limited to the fraction of cultivable bacteria. The big advantage of studies on isolates is that the ecology of the plasmid's host and the plasmid's function can be assessed. The presence of plasmids was often revealed as a result of genome sequencing projects as in the case of the citrus pathogen *Xylella fastidiosa* (Marques et al., 2001). However, it is known that only a rather small proportion of environmental bacteria are accessible to cultivation. Thus, to gain a more complete picture,

cultivation-independent methods are increasingly used to detect and quantify plasmid abundance in the phytosphere (Smalla et al., 2015). By extracting the TC-DNA from the phytosphere, the plasmids in the whole bacterial population present in a representative sample type can be studied. However, plasmids typically are present only in a small fraction of the bacterial community and in low abundance (Heuer et al., 2008). Thus, to detect plasmids in TC-DNA by PCR-amplification using primers for conserved regions, Southern blot hybridization is required to increase the sensitivity and specificity of plasmid detection (Dealtry et al., 2014). Quantitative real-time PCR allows the quantification of plasmids in a microbial community, and its relative abundance is provided by relating the plasmid-specific gene copy numbers to the 16S rRNA gene copy number, as it was done with *trfA* and *korB* for IncP-1 plasmids (Heuer et al., 2012; Jechalke et al., 2013a). To our knowledge, recently developed tools to study plasmid occurrence and diversity by directly sequencing the plasmidome (Li et al., 2012) or by determining the potential plasmid host by EPIC-PCR (Spencer et al., 2015) were not yet employed for phytosphere-associated bacteria. The diversity of IncP-1 plasmids was recently studied by amplicon sequencing of the *trfA* gene (Dealtry et al., 2014), but this approach was not yet used to study the IncP-1 plasmid diversity in the phytosphere.

Additionally, transferable plasmids can be captured independently from the cultivability of their original hosts. Different plasmid-capture approaches are known, e.g. the *in vitro* transposon-aided capture method (TRACA). An EZ-*Tn5 OriV* Kan2 transposon is inserted (Jones and Marchesi, 2006), and no selectable markers, conjugative function or mobilization are needed. Interestingly, plasmids captured by means of exogenous plasmid isolations via biparental or triparental mating from the rhizosphere of a wide a range of crops such as wheat (van Elsas et al., 1998), alfalfa (Schneiker et al., 2001), maize and grass (Jechalke et al., 2013c), but also from the mycosphere (Zhang et al., 2014) were often affiliated to BHR plasmids of the IncP-1 or the pPromA group. There is no doubt that the progress made in next generation sequencing technologies strongly improved our knowledge of the diversity of plasmids in bacteria colonizing the phytosphere. These tools are of outmost importance to understand the contribution of plasmid-encoded traits to foster the adaptation and diversification of their hosts. A list of selected techniques and their advantages and disadvantages is given in Table 1.

*Bioinformatic approaches*

Different sequencing techniques are currently available for the characterization of plasmids. With the sequencing by PacBio, comparatively fast sequencing with a very long read length is possible (Rhoads and Au, 2015). The long reads facilitate the plasmid sequence assembly. Even though the PacBio method is hindered by a relatively low throughput and high error rate, this sequencing approach might be complemented with the Illumina approach. This combination enabled the sequencing of the natural plasmid pMM259 that mediates genetic exchange by HGT between rhizobia and *Roseobacter* species (Bartling et al., 2017). Furthermore, whole genome sequencing by PacBio revealed a plasmid in the biocontrol strain *Bacillus amyloliquefaciens* subsp. Plantarum S499 (Molinatto et al., 2016), which was later assumed to be important for root colonization and control of surfactant production, as well as biofilm formation (Molinatto et al., 2017). Plant pathogenic bacteria were studied by PacBio as well, e.g. Lu et al. (2018) revealed the presence of pathogenicity-related plasmids in *Clavibacter michiganensis* subsp. *insidiosus* which can cause crop diseases. The megaplasmid found in *Ralstonia solanacearum* contains genes for type II and III secretion systems (Li et al., 2018). Another rather new sequencing possibility is the nanopore sequencing technology, which enables the sequencing of single DNA molecules. The available technologies are reviewed in more detail in Goodwin et al. (2016).

Nowadays, many bioinformatics tools are available for different plasmid analyses. The first step in the analysis is the distinction between chromosomal and plasmid DNA. Plasmids can be identified using tools such as cBar, plasmidSPAdes, PlasmidFinder or MLST (Arredondo-Alonso et al., 2016; Carattoli et al., 2014; Zhou and Xu, 2010). The reads can be mapped to a reference database using SRST2 (Inouye et al., 2014). Plasmid reconstruction methods are very useful to analyze plasmids by clustering dendrograms, allowing the comparison of plasmid diversity and adaptation (de Toro et al., 2014). Examples for available tools include PLACNET, Recycler and plasmidSPAdes (Antipov et al., 2016; Lanza et al., 2014; Rozov et al., 2016). Reconstructed plasmids can be investigated for potential functions, such as the presence of resistance genes (Clausen et al., 2016; Gupta et al., 2014; McArthur et al., 2013; Zankari et al., 2012). Several of the applications were reviewed in Orlek et al. (2017) and Edwards and Holt (2013).

**Table 1:** Advantages and disadvantages of selected tools for the investigation of plasmids in the phytosphere.

| Methods | Advantages | Disadvantages |
|---|---|---|
| Plating of environmental bacteria followed by plasmid extraction | ○ Easy to perform<br>○ Focus on markers possible by selective plating | ○ Limited to the cultivable fraction of bacteria<br>○ Not all environmental bacteria are suitable for plasmid extraction |
| PCR-amplification of known backbone genes from total community DNA | ○ Represents the whole community<br>○ Combined with Southern blot hybridization it allows a more sensitive analysis | ○ Original plasmid host remains unknown<br>○ Limited detection of novel sequences |
| Quantification of plasmids relative to 16S gene copy number by quantitative real time PCR | ○ Determination of plasmid abundance, e.g. extracted from total community DNA<br>○ More specific than PCR e.g.by use of additional TaqMan probe | ○ Original plasmid host remains unknown<br>○ More expensive than PCR |
| Pyrosequencing | ○ Detection of unknown plasmids/sequences, even when the host is unknown | ○ Difficult assembly<br>○ Not state-of-the-art method |
| Exogenous plasmid isolation by bi- or triparental mating | ○ Independent from original host and its cultivability<br>○ Possibility to capture novel plasmids | ○ Original plasmid host remains unknown<br>○ Depends on selectable markers<br>○ Depends on replication in recipient and transferability of plasmid |
| Transposon aided capture method (TRACA) | ○ Culture-independent<br>○ Does not rely on plasmid-encoded traits<br>○ Possibility to capture novel plasmids | ○ Not all captured plasmids are stable in *E. coli*<br>○ Large plasmids are difficult to capture due to low copy number and challenging transformation |
| GFP-tagged plasmids | ○ Easy quantification and localization of the plasmids<br>○ Offers potential to discover original hosts/new hosts/ transfer rate etc | ○ Might be difficult to transfer<br>○ Potential metabolic burden |
| Illumina sequencing | ○ Rapidly decreasing costs<br>○ High throughput | ○ Short read length |
| PacBio sequencing | ○ Very long read length | ○ Low throughput |
| Nanopore sequencing | ○ Single molecule sequencing<br>○ Potential of long read length<br>○ Low cost | ○ Single-base resolution potentially reduced by stochastic motion of the DNA molecule |

*Plasmid transfer, maintenance and evolution studies*
The transfer and distribution of plasmids can also be investigated *in situ* via incorporation of marker genes. In this approach, *gfp*-tagged plasmids under the control of a *lac* promoter are used together with donor bacteria that have a chromosomally inserted *lacIq* repressor. While there is no *gfp* transcription in the donor cell, once the plasmid enters the recipient bacterium, the *lac* promoter is not under the control of the repressor anymore and the *gfp* is expressed (Klümper et al., 2015). By fluorescent microscopy or flow cytometry, plasmid transfer can be quantified and transconjugants isolated. Such approach was already applied for the rhizosphere community of barley (Musovic et al., 2006). Labeling of plasmids with marker genes like *gfp* allows the investigate of their transfer and also their stability. Plasmid transfer can be investigated through growth of the recipient on selective media (Bale et al., 1988). However, this method is limited to the cultivable fraction of a bacterial community.

Most of the studies on the cost and benefits of plasmids for their hosts were done in rich nutrient broth. However, these studies might provide only little insights into plasmid cost and benefits for bacteria residing in natural ecosystems. Although it is well documented that a subset of soil bacteria is enriched in the phytosphere, the generation times are assumed to be much longer and thus plasmid carriage might be less costly. Recently, Bziuk et al. (unpublished) examined the potential fitness cost or benefit of the IncP-1 plasmid pTL25 that was captured by triparental mating from the rhizosphere of lettuce grown in non-polluted soil. A competition experiment was performed by inoculating lettuce plants grown in soil microcosms with plasmid-carrying and plasmid-free *Pseudomonas putida* KT2442 (50:50 ratio). While a stable maintenance of pTL25 carrying *P. putida* KT2442 was observed in the rhizosphere, its proportion in bulk soil decreased. These findings indicate that in the rhizosphere carriage of plasmid pTL25 seemed not to be disadvantageous.

Plasmid evolutionary studies might answer the question of the contribution of some plasmids to the versatility of plant-associated bacteria. Plasmid evolution occurs mainly through cost amelioration and stability improvement (Hughes et al., 2012). These adaptations of plasmids to their host were described previously (Bouma and Lenski, 1988). Nevertheless, evolutionary studies in the phytosphere are still scarce.

**Conclusions**
Over the past decades an increasing number of studies on plasmids in the phytosphere increased our knowledge of their diversity and functions in the plant environment. In general, plasmids in plant associated bacteria facilitates their survival in the phytosphere environment, with the rapidly changing conditions and diverse biotic and abiotic stresses. Plasmid-encoded traits can contribute to an improvement of plant growth and health. However, an increased ecological understanding of plasmids, related functions and their respective hosts is needed for the development of new crop breeding strategies. Hence, plasmid-encoded functions could help the plant to better respond to biotic and abiotic stresses.

In addition, plasmids are also important carriers of plant-pathogenic traits. To improve disease prevention, it is essential to investigate their function, virulence and fitness contribution, as well as their host specificity. While the microbiome of different crops is presently studied in detail, systematic studies on their plasmidome were not yet undertaken. Furthermore, the presence of pollutants such as pesticides, veterinary medicines, metal compound introduced via organic fertilizers or irrigation water can foster the abundance of bacteria carrying plasmids with antibiotic or heavy metal resistance genes. In particular, the role of co-selection of antibiotic resistance by heavy metal or disinfectant compounds needs further investigation. The hidden diversity of plasmids in the rare phytosphere microbiome can only be assessed by a polyphasic approach comprising isolates, the molecular analysis of TC-DNA from detached cells or from enrichments and exogenously captured plasmids. These approaches would allow to detect antibiotic resistance plasmids in enteric bacteria that are typically not very abundant in the phytosphere such as *E. coli* or *Salmonella.* These might be taken up by humans via raw fruits and vegetables and present risks for human health.

Rapid developments in next generation sequencing techniques offer the ability to sequence plasmids from plant-associated bacteria or plasmids captured into recipients. However, due to their low abundance, the direct sequencing of plasmids extracted from the phytosphere is still difficult. Nevertheless, a large number of plasmid sequences are already available. These allow comparative studies on plasmids and the development of tools for systematic studies on the occurrence and

diversity in the phytosphere. However, it remains a challenging but very important task to investigate the cost and benefit of plasmids for their host bacteria in the phytosphere in order to understand the role of plasmids in interactions between bacteria and plants.

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