Mechanisms of bacterial attachment to roots.

A common biphasic model of root attachment exists in agriculturally important microbial species.

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Abstract

The attachment of bacteria to roots constitutes the first physical step in many plant-microbe interactions. These interactions exert both positive and negative influences on agricultural systems depending on whether a growth-promoting, symbiotic, or pathogenic relationship transpires. A common biphasic mechanism of root attachment exists across agriculturally important microbial species, including *Rhizobium*, *Agrobacterium*, *Pseudomonas*, *Azospirillum* and *Salmonella*. Attachment studies have revealed how plant-microbe interactions develop, and how to manipulate these relationships for agricultural benefit. Here, we review our current understanding of the molecular mechanisms governing plant-microbe root attachment and draw together a common biphasic model.

Keywords: root attachment, plant-microbe interaction, rhizobacteria, adhesion, molecular mechanism, plants

Introduction

The rhizosphere is the zone of soil immediately surrounding plant roots and can support a rich and diverse bacterial community (Walker 2003). Plants may release up to a fifth of their photosynthate via their roots (Estabrook 1998), and so the rhizosphere is under strong influence of root exudates and the secreted products of bacterial metabolism and degradation. Attaching to the rooting system not only enables plant-microbe interactions to develop, but also carries the significant benefit of anchoring bacteria in the nutrient-rich environment of the rhizosphere. Root exudation is a critical driving force shaping the establishment of microbial populations (Grayston and Campbell 1996). Exudates can serve as preferential growth substrates for particular microbes, and in turn, the interactions that develop with these microbes can exert preferential selectivity on plant fitness through their positive or negative growth influences.

Reviewing root attachment in agriculturally important microbial genera, *Rhizobium, Agrobacterium, Pseudomonas, Azospirillum* and *Salmonella*, demonstrates a common biphasic mechanism exists within them. This comprises a primary attachment phase, characterized by a weak, reversible and non-specific binding of bacteria to the root surface. Followed by a secondary attachment phase, characterized by a switch to a stronger, more specific binding mode, and the synthesis of extracellular fibrils aiding further bacterial accumulation and aggregation. Divisions of this biphasic mechanism are denoted differently throughout the literature. Primary attachment is commonly referred to as a 'settlement', 'adsorption' or 'reversible' phase, and secondary attachment is commonly referred to as a 'residence', 'anchoring' or 'irreversible' phase (Matthyssee 2014; Michiels, et al. 1991; Rodríguez-Navarro, et al. 2007).

Bacteria exhibiting this biphasic root attachment mechanism encompass a diverse range of plant interactions, as plant-growth promoters, biofertilisers and pathogens. One factor common to the development of these plant-microbe interactions is the initial requirement to attach to plant roots. In some cases, the attached bacteria reside adhered to the outer surface of the root, or in other cases, attachment is a prerequisite for the endophytic or pathogenic colonisation of the inner root. It is important to note that the vast majority of root attachment research has been carried out in plant-microbe interactions involving Gram-negative bacteria (Bogino, et al. 2013; de Jesus Sousa and Olivares 2016; Rodríguez-Navarro, et al. 2007), such as the five well-characterised genera of *Rhizobium, Agrobacterium, Pseudomonas, Azospirillum* and *Salmonella*. Gram-positive bacteria, such as members of the *Bacillus* and *Streptomyces* genus, also engage in agriculturally important plant interactions, and have more recently become a bigger focus of attention in root attachment research, having already been generally well-characterised in abiotic biofilm formation processes (Beauregard, et al. 2013; Habib, et al. 2017; Viaene, et al. 2016).

Rhizobium, *Agrobacterium* and *Azospirillum* are grouped within the class of alpha-proteobacteria. *Rhizobium* species are able to engage in symbiosis with leguminous plants and function as a nitrogen biofertiliser. After attaching to and colonising legume roots, rhizobia become internally housed in protective 'nodule' structures, within which they catalyse biological nitrogen fixation and provide the plant with useable nitrogen sources for growth (Udvardi 2013). They receive carbon sources primarily in the form of dicarboxylates in return, the metabolism of which helps fuel the energetically expensive process of nitrogen fixation (Udvardi 2013). Many species of *Agrobacterium* are non-pathogenic and live saprophytically in the soil (Danhorn and Fuqua 2007). However, the best-studied species, *Agrobacterium tumefaciens*, is the causative agent behind crown-gall disease in many flowering plants, and the nature of this pathogenic DNA transmission gives it significant uses as a tool for genetic transformation.

Azospirillum is probably the best-characterised genus of plant growth-promoting rhizobacteria (PGPR) (Steenhoudt and Vanderleyden 2000). *Azospirillum* species associate with numerous agriculturally important plants and positively influence plant growth, crop yields and nitrogen-content (Steenhoudt and Vanderleyden 2000). Plant growth promotion is attributed to several mechanisms, including the production of growth phytohormones, such as auxins, cytokyinins and gibberellins, and biological nitrogen fixation. Growth phytohormones commonly improve plant growth by stimulating root hair growth and increasing the root surface area, which improves the acquisition of water and mineral nutrients (Steenhoudt and Vanderleyden 2000). Azospirilla are able to exist as free-living nitrogen-fixing bacteria, but can also attach to roots, and some species can colonize the internal root tissues as endophytes. Intriguingly, *Azospirillum* may represent an evolutionary bridge between water- and soil-dwelling microbes [4], and a better understanding of how *Azospirillum* colonises plants might therefore also help improve understanding of how microbes transferred from water to soil environments (Orlandini, et al. 2014).

Pseudomonas and *Salmonella* are both genera of gamma-proteobacteria, they exhibit extensive species diversity and are capable of colonizing a wide range of niches (Madigan and Martinko 2005). The best-studied rhizosphere pseudomonads are *Pseudomonas fluorescens, Pseudomonas syringae* and *Pseudomonas putida*. From an agricultural perspective, *P. fluorescens* and *P. putida* are valued for their plant growth-promoting properties, including growth phytohormone production, plant pathogen suppression, iron chelation by siderophore production and aiding in bioremediation (Goswami, et al. 2013). Conversely, strains of *P. syringae* are known for plant pathogenicity and their ability to infect a diverse range of plant species. Following attachment and infection, the pathogenic action of the majority of *P. syringae* strains is to secrete plant toxins and produce ice nucleation proteins, which cause water to freeze and induce ice injury to plant cells. *Salmonella* are commonly known for pathogenicity in animal species, but these microbes can also live saprophytically in soil and infect plants. By infecting plants, *Salmonella* are later able to transfer infection to animals through plant ingestion (Barak, et al. 2005; Brankatschk, et al. 2014; Wiedemann, et al. 2015). There are numerous reports linking *Salmonella* contaminated plant produce to food poisoning in humans (Wiedemann, et al. 2015; Krtinić, et al. 2010), yet the root attachment mechanism of *Salmonella* remains poorly characterised.

Molecular mechanisms governing primary attachment

Root migration

Migration towards the root via chemotaxis constitutes an attraction phase that generally precedes attachment (Begonia and Kremer 1994). Root-exuded nutrients often serve as chemoattractants. Common chemoattractant signals include phenolics, organic acids, amino acids and phytosiderophores. Flavonoids induce positive chemotaxis in strains of *Rhizobium* (Aguilar, et al. 1988; Armitage, et al. 1988; Dharmatilake and Bauer 1992), and malate and benzoates induce positive chemotaxis in strains of *Azospirillum* (Lopez-de-Victoria and Lovell 1993). Flagella and pili are filamentous proteinaceous appendages that extend from the bacterial cell surface. Bacteria typically use flagella or pili as propellers to migrate towards the root and position within sufficient proximity for initial weak attractive forces to develop (Berne, et al. 2015; Caetano-Anollés, et al. 1988; Zheng, et al. 2015). This active propulsion helps overcome any repulsive barriers, such as repulsive electrostatic forces due to the electrostatic charge of the cell envelope (Berne, et al. 2015). Flagella are significantly longer than pili and are present at fewer numbers per cell, they generally show a distinct pattern of distribution (i.e. polar flagellum) whereas pili are distributed randomly over the cell surface (Van Gerven, et al. 2011). In addition to a role in motility, flagella and pili can also function adhesively in attachment (Croes, et al. 1993; Pratt and Kolter 1998; Smit, et al. 1989; Van Gerven, et al. 2011; Vesper 1987; Zheng, et al. 2015).

Fundamental weak binding forces

The universal characteristics of primary attachment are weak, reversible, and non-specific binding, allowing association of single cells to the surface. Primary attachment is initially dictated by physiochemical and electrostatic attractions between surface molecules of the root and bacterial cell envelope (Berne, et al. 2015). A number of fundamental weak binding forces form these preliminary surface associations across all plant-microbe interactions: Van der Waals forces, electrostatic forces and hydrophobic interactions (Berne, et al. 2015; Van Loosdrecht, et al. 1987). Van der Waals forces are the ubiquitous attractions that arise from momentary changes in electron density in a molecule (Kendall and Roberts 2015). They are displaced by stronger electrostatic forces, caused by differences in electric charge between molecules (Berne, et al. 2015; Van Loosdrecht, et al. 1987). The hydrophobic properties of the microbial cells and root surface contribute the strongest force of these initial associations, such that hydrophobic cells experience attractive forces to hydrophobic surfaces (Giaouris, et al. 2009; Kochkodan, et al. 2008; Krasowska and Sigler 2014).

The bacterial cell surface possesses a net negative charge under the majority of physiological conditions (Poortinga, et al. 2002). Protrusive components of the cell surface initially function in primary attachment by means of their electrostatic charge and steric properties (Janczarek, et al. 2015). Cell surface electrostatic properties are modified by contact of the cells with inert

surfaces such as soil particles (Bushby 1990), and unsurprisingly, the heterogeneity of electrostatic cell surface distribution also affects repulsion during bacterial adhesion (Poortinga, et al. 2002). However, electrostatic surface properties are only important in the short-term (Bogino, et al. 2013; Janczarek, et al. 2015; Poortinga, et al. 2002; Van Loosdrecht, et al. 1987), and contribute relatively weak interactions towards the overall efficiency of primary attachment. This is exemplified well with exopolysaccharides (EPS) coating rhizobial cells. EPS electrostatic charge plays a minor role in overall mechanics of initial physiochemical attachment compared to the much stronger forces of EPS hydrophobicity (Janczarek, et al. 2015), which in turn, plays a minor role comparative to adhesive binding mediated by any microbe-specific binding factors later in the attaching mechanism.

Microbe-specific primary attachment factors

Primary attachment in *Rhizobium*

Microbe-specific primary attachment factors, such as outer surface proteins, polysacharrides and flagella, form stronger yet still reversible binding associations with the root (Fig. 1). Within a genus, bacteria can display a range of species-specific and even strain-specific attachment and colonization mechanisms (Berne, et al. 2015; Steenhoudt and Vanderleyden 2000). Attachment is affected by a range of soil and root physiochemical properties; such as pH, Ca^{2+} and Mg^{2+} concentrations, water availability and pre-treatment of roots (Rodríguez-Navarro, et al. 2007). This is exemplified well in *Rhizobium leguminosarum,* a rhizobial species that forms nitrogen-fixing symbioses with legumes such as pea, vetch, lentil and faba bean. *R. leguminosarum* utilizes primary attachment mechanisms dependant on soil pH (Fig. 1). In acidic soil, the rhizobial surface polysaccharide glucomannan binds root-hair expressed lectin (Laus, et al. 2006). Lectins are carbohydrate-binding proteins, found ubiquitously in nature, that function as recognition molecules in cell-cell interactions across a wide variety of biological systems to mediate binding in a reversible and highly specific manner (Lagarda-Diaz, et al. 2017; Sharon and Lis 2004). This lectin-mediated mechanism of primary attachment is also used in *Bradyrhizobium japonicum* attaching to soybean roots (Lodeiro and Favelukes 1999; Lodeiro, et al. 2000). Considering the phylogenetic separation between *B. japonicum* and *R. leguminosarum* and the high lectin content in leguminous species (Lagarda-Diaz, et al. 2017), this lectin-mediated attachment mechanism is likely utilised in other *Rhizobium*-legume symbioses too.

Under alkaline conditions, root lectins are solubilised, and bacterial polysaccharides such as glucomannan no longer bind (Laus, et al. 2006). The mechanism of attachment under alkaline and neutral pH might involve an extracellular rhizobial protein ‘rhicadhesin’, although this is unproven. Rhicadhesin was postulated to mediate attachment between the rhizobial cell and root hair surface in a calcium(Ca^{2+})-dependent manner, and similarly to the dispersal of plant lectin under alkaline pH, rhicadhesin is suggested to disperse under acidic

pH. Low Ca^{2+} conditions produce a significant reduction in the attachment of rhizobia to pea root-hair surfaces (Smit, et al. 1987), and this is believed to be due to the presence of a Ca^{2+}-dependant attachment protein. Rhicadhesin was reported to be a 14–kDa Ca^{2+}-binding protein that inhibits rhizobial attachment to pea and is secreted by cells growing in a low-[Ca^{2+}] medium (Smit, et al. 1989). However, 30 years past these original findings, neither the gene encoding rhicadhesin nor the protein itself has been identified. It is possible multiple copies of the gene exist, and this might explain difficulties in its identification (Matthyssee 2014), but the role rhicadhesin plays in attachment remains unclear (Downie 2010; Matthyssee 2014). It can be argued that because the protein has been defined by its ability to inhibit attachment, this certainly supports a role for such a protein in the stability and structure of the cell outer surface, but not necessary a direct role in attachment (Matthyssee 2014). More evidence is needed to better define a role for a rhicadhesin in attachment, and this requires identification of the encoding gene(s). This should now be possible by peptide sequencing of the 14 kDa fraction of rhizobial surface proteins.

Primary attachment in *Agrobacterium*

The importance of characterising root attachment in *A. tumefaciens* is highlighted by the strong correlation between attachment to root cells and susceptibility of the plant to crown gall disease (Hawes and Pueppke 1987), and that direct physical contact via attachment is essential to facilitate DNA transfer into cells (Penalver, et al. 1996). *Agrobacterium* possess a very closely related polysaccharide to glucomannan in the form of a unipolar polysaccharide (UPP) adhesin. However, a key difference between the two is that UPP mediates an irreversible polar attachment in *A. tumefaciens,* and glucomannan mediates a reversible lectin-receptor attachment in *R. leguminosarum* (Matthyssee 2014; Tomlinson and Fuqua 2009). The irreversible association mediated by UPP gives it a role in secondary attachment, and primary attachment is facilitated by a combination of surface proteins, molecular adhesins, pili and capsular polysaccharides (Rodríguez-Navarro, et al. 2007). The Ca^{2+} adhesion rhicadhesin is also proposed to function in primary attachment in *A. tumefaciens* (Smit, et al. 1989; Swart, et al. 1993), however, genetic or biochemical support is yet to be shown and so similarly to rhizobia a defined role remains unclear.

The Ti or ‘tumour inducing’ plasmid of *Agrobacterium* contains a large part of the genetic equipment for transferring DNA to plant cell (Christie and Gordon 2014). On this plasmid are virulence ‘*vir’* genes essential to gene transfer during plant infection, and within this group is the VirB operon that plays a role in primary attachment (Tomlinson and Fuqua 2009). VirB forms the T-pilus that functions as an adhesive pili and initiates physical contact with the root surface (Fig. 1) (Backert, et al. 2008; Christie, et al. 2005; Fullner, et al. 1996). VirB localizes at the cell poles and the T-pilus is visualized to extend from these poles (Atmakuri, et al. 2007; Lai and Kado 2000); this system mediates polar attachment (Tomlinson and Fuqua 2009). However, the T-pilus is not essential for attachment as strains unable to produce this structure or even deficient in the Ti plasmid entirely are still able to attach to plant tissue surfaces in an indistinguishable manner from the wild-type (Danhorn, et al. 2004; Ramey, et

al. 2004; Tomlinson and Fuqua 2009). This points to the presence of attachment elements encoded separately from the Ti plasmid, such as chromosomally encoded polysaccharides and adhesive proteins (Reuhs, et al. 1997). Intriguing, the cryptic plasmid (pAT) of *A. tumefaciens* is not required for attachment or virulence of the strain, but mutation of two genes (attachment 'att' genes *attC* and *attG*) on the plasmid has a dominant negative effect that obliterates primary attachment, causing the bacteria to become avirulent and non-attaching on both carrot and tomato plants (Matthysse, et al. 2008). The mechanism by which attachment and virulence is blocked by these mutations is unclear (Matthysse, et al. 2008).

Primary attachment in *Azospirillum*

The majority of *Azospirillum* strains can associate with the root surface but only a few strains are able to colonise the inner root as endophytes (Döbereiner, et al. 1995; Patriquin, et al. 1983). The importance of attachment in PGPR interactions is well studied in *Azospirillum* (Rodríguez-Navarro, et al. 2007). Within this genus, the majority of research is focused on *Azospirillum brasilense* and its' ability to promote the growth of numerous agriculturally important crop plants, including cereals such as wheat. Whilst azospirilla are able to promote plant growth as free-living soil bacteria by secreting plant phytohormones into the rhizosphere, their attachment to roots maximises their mutual benefit (Burdman, et al. 2000).

The primary attachment phase of *Azospirillum* is commonly referred to as the 'adsorption phase'. The polar flagellum of *A. brasilense* is required for initial adsorption to roots, alongside it's role in motility (Fig. 1)(Croes, et al. 1993; Mora, et al. 2008; Rodríguez-Navarro, et al. 2007). Flagella-deficient mutants are impaired in attachment to wheat roots, and the purified polar flagella binds directly to the wheat root surface (Croes, et al. 1993). Flagellin is a major component of the polar flagellum. The glycosylation of flagellin is suggested to be carried out by the same genes involved in the biosynthesis of the sugars in lipopolysaccharide (LPS), another cell surface moiety (Rossi, et al. 2016). Mutation of these genes affects the structure of polar flagellin, the production of LPS and the primary attachment abilities of *A. brasilense* (Rossi, et al. 2016). Outer-membrane proteins (OMPs) are involved in both the primary and secondary attachment phases in *Azospirillum* (Fig. 1; Fig. 2). OMPs are constitutively present as surface-exposed proteins and involved in initial adsorption onto the root, then their surface-exposed domain enable them to function in cell aggregation through interaction with the surface domains of neighbouring bacteria (Burdman, et al. 2001). This mode of action is most well-characterised for the 47.7 kDa major OMP of *A. brasilense* (Burdman, et al. 1999).

Primary attachment in *Pseudomonas*

Models of primary attachment in pseudomonads are relatively poorly characterised compared to later stage secondary attachment. Early research implicates pili have a role in primary attachment in *P. fluorescens* (Vesper 1987) (Fig. 1). However, the nature of the attachment assay means this may be at least partly be due to the importance of pili in motility, opposed to a direct, physical role in attachment itself (Vesper 1987). Both *P. fluorescens* and *P. putida* flagella mutants have colonization defects on plant surfaces, but again, it is possible this is due to the role of flagella in motility opposed to a direct physical role in attachment. Direct flagella binding studies need to be carried out to clarify this involvement. Initial attachment to plant roots produces a global change in gene expression in *P. putdia,* and isolation and characterization of these genes shows similarities to those involved in the colonisation of abiotic surfaces (Sauer and Camper 2001). Structural components of flagella, type IV pili and genes involved in polysaccharide biosynthesis are all upregulated in primary attachment (Sauer and Camper 2001).

Outer membrane porin F (OprF) is a major outer membrane porin of pseudomonads and is believed to function as a root adhesion protein in *P. fluorescens* (De Mot and Vanderleyden 1991). OprF demonstrates adhesive properties to wheat, barley, maize and sunflower in short time scale assays of one hour that suggest it functions in primary attachment (Fig. 1), as this is likely too brief a period for tighter secondary attachments to develop (De Mot and Vanderleyden 1991). OprF also demonstrates adhesiveness to cucumber and tomato roots (Crespo and Valverde 2009). The number of *oprF* mutants able to loosely adhere to the roots is significantly lower than the wild-type (Crespo and Valverde 2009), with this loose adherence again implicates a role in primary attachment. Decreased tight adherence to the roots is also observed (Crespo and Valverde 2009), perhaps because reduced primary attachments limits secondary attachments, or because OprF functions in both phases of the attachment mechanism. It is unclear exactly how OprF binds to the root surface and the root surface components it associates with.

Primary attachment in *Salmonella*

Salmonella is widely known for infecting animal species, and *Salmonella* food poisoning in humans is generally believed to be associated with consuming infected animal produce such as meat or eggs. However, *Salmonella* is also able to attach to numerous agriculturally important crop species, the consumption of which can directly infect the grazing animal species and humans (Hernández-Reyes and Schikora 2013; Wiedemann, et al. 2015). The root attachment mechanism of *Salmonella* is poorly characterized, particularly in comparison to characterisation of the direct infection of animal species (Hernández-Reyes and Schikora 2013; Wiedemann, et al. 2015). It is believed *Salmonella* is transferred to soil through treatment with a natural, contaminated fertiliser source (such as animal manure) or through contaminated water irrigation (Krtinić, et al. 2010). The percentage of *Salmonella* outbreaks

associated with the consumption of contaminated plant-produce is on the rise (Barak, et al. 2008; Gould, et al. 2013; Nygård, et al. 2008; Zheng, et al. 2013), and it is only just becoming apparent the extent to which better understanding this plant-microbe interaction is required.

Salmonella enterica is found in the rhizosphere of numerous crop plants (Berg, et al. 2005). The bacteria exhibits positive chemotaxis towards the root exudates of lettuce and microarray analysis indicates pathogenicity-related genes are induced (Klerks, et al. 2007). Flagella are important for the root attachment of *Salmonella typhimurium*; flagella mutants are unable to invade the root junctions of *Arabidopsis thaliana* plants (Cooley, et al. 2003). It is possible this decreased attachment is a result of reduced motility, as opposed to a direct physical role of the flagella in surface adherence. It is unclear which phase of root attachment the flagella may function in, although from the understanding of other rhizobacteria it seems most likely flagella will be involved in initial primary attachment, and then potentially play a role in secondary attachment too.

A transposon mutant screen to identify *S. enteric* genes with a role in attachment to alfalfa roots sheds more light on the root attachment capabilities of *Salmonella* (Fig. 1) (Barak, et al. 2005). Twenty mutants with reduced adherence were identified, thirteen of which are uncharacterized genes (Barak, et al. 2005). Identified genes with a probable role in primary attachment are within an aggregative fimbral (*agf*) operon, encoding proteinaceous surface fimbriae with the ability to function as bacterial adhesions, and a stationary-phase sigma factor (RNA polymerase, sigma S; *rpoS*) with a number of regulatory roles, including regulation of the *agf* fimbral operon and other adhesive factors such as *Salmonella* pili (Barak, et al. 2005). Similarly to flagella and pili, fimbriae are filamentous proteinaceous structures that extend from the bacterial cell surface. Fimbriae are shorter and more numerous per cell than pili, and unlike flagella and pili, no do function in active motility (Berne, et al. 2015; Van Gerven, et al. 2011). Fimbriae and pili are also important factors in the animal pathogenicity of *Salmonella*, and appear to be relatively common primary attachment factors in numerous rhizobacteria. At least thirteen fimbral operons are present in the genome of *S. typhimurium*, and it is probable some of these also function in plant root attachment but are yet to be characterised.

Molecular mechanisms governing secondary attachment

Secondary attachment is characterised by bacteria binding in a tight and irreversible manner to the roots. Typical of this switch to a stronger and more specific binding mode is the synthesis of extracellular fibrils aiding accumulation of bacterial aggregates and the formation of microcolonies on the root (Fig. 2) (Downie 2010). For many root-attaching bacteria, the synthesis of secondary attachment factors is induced in response to successful primary attachment (Rodríguez-Navarro, et al. 2007). Many microbes use secondary attachment simply as a means of secure adhesion to the root surface, while for others it may be a pre-

requisite of endophytic root colonisation, pathogenic infection or in the case of *A. tumefaciens*, genetic transfer.

Secondary attachment in *Rhizobium*

Nitrogen-fixing rhizobia exhibit accumulation of additional bacteria at the site of initial adhesion and the synthesis of extracellular fibrils largely constructed from cellulose (Fig. 2). Rhizobia attach specifically to the tips of developing root hairs, a region distinct from the epidermal surface of the root. Root hair binding is important as the first step in legume infection, leading to nodule formation and biological nitrogen fixation (Gage 2004; Smit, et al. 1986). Two types of nodules exist, determinant and indeterminant. Determinant nodules are found on a number of tropical legumes and lose meristematic activity shortly after formation to produce spherical mature nodules. Indeterminant nodules are found on the majority of other legumes and maintain an active meristem that continues to generate new cells; this produces more cylindrical mature nodules separated into distinct developmental zones.

Tight secondary attachment allows infection thread formation when rhizobia become trapped between two root hair cells walls after nodulation signalling induces curling of the root hair (Gage 2004). Extracellular fibrils aid formation of bacterial aggregates as a 'cap' on the root hair, a structure that can be likened to a biofilm, but has a very distinct attachment process compared to biofilm formation on an inert surface (Laus, et al. 2006; Smit, et al. 1987; Williams, et al. 2008). Secondary attachment factors in *Rhizobium* include cellulose fibrils, polysaccharides and secreted proteins. These secreted proteins commonly contain *Rhizobium*-adhering or Cadherin-like domains such as the *Rhizobium*-adhering protein (Rap) family (Poole 2017). Cellulose fibrils help facilitate tight adherence between rhizobial cells as they form a cap on the root hair (Poole 2017), but are not essential for symbiosis. In *R. leguminosarum*, mutants deficient in cellulose fibril production are still able to form nodules (Smit, et al. 1987). It is likely cellulose fibril formation helps facilitate the colonization of fast-growing root hairs but is not strictly essential for the symbiosis (Laus, et al. 2006).

A polysaccharide that plays a significant role in secondary attachment is EPS. EPS is a major component of the cell surface and composed of carbohydrate polymers that demonstrate highly variable composition between strains and species (Downie 2010). Polymers are formed from linear or branched molecules of either a homopolysaccharide sugar or different sugars as a heteropolysaccharide (Balsanelli, et al. 2014). EPS helps facilitate attachment and cellular aggregation on both abiotic and biotic surfaces. EPS deficient mutants of indeterminate-nodule forming *Rhizobium leguminosarum* have defective root attachment and strongly reduced numbers of infection foci (Williams, et al. 2008). The EPS of *R. leguminosarum* is known as acidic EPS (Bogino, et al. 2013), and is composed of octasaccharide repeating units of D-glucose, D-galactose, and D-glucuronic acid residues in a 5:1:2 molar ratio (Breedveld, et al. 1993; O'Neill, et al. 1991; Philip-Hollingsworth, et al. 1989; Robertsen, et al. 1981). A

R. leguminosarum acidic EPS mutant has an interesting phenotype of near-zero attachment and cap formation at root hairs, but is still able to attach along the boundaries of adjacent root epidermal cells (Williams, et al. 2008). This suggests that whilst acidic EPS is required for attaching to root hairs, it is not essential for attaching at the boundaries of root epidermal cells (Williams, et al. 2008). The differing structural physiologies of the root hair versus root epidermis leave them receptive to different attachment mechanisms.

The PrsDE Type I protein secretion system is responsible for the export of several secondary attachment proteins in *R. leguminosarum*, including Raps (RapA1, RapA2, RapB, RapC and RapD) and EPS-glycanases (PlyA and PlyB) (Fig. 2) (Russo, et al. 2006). EPS-glycanases cleave cell surface EPS, and *plyB*/*plyA* mutants produce an EPS of considerably greater length than the wild-type (Russo, et al. 2006). Correct EPS processing is important for attachment in *Rhizobium*. Secretion mutants of *prsD* and *prsE* are unable to develop secure secondary attachment cell aggregations (Russo, et al. 2006). The *rapA1* gene is the most well-characterised Rap group protein, and encodes a 30-kDa Ca^{2+}-binding protein that localizes to the cell pole and promotes cellular aggregation (Russo, et al. 2006). Rap adhesins are believed to function in agglutination through binding either EPS or capsular polysaccharide (Russo, et al. 2006). The over-expression of RapA1 in *R. leguminosarum bv. trifolii* enhances attachment to its host legume by up to five-fold (Mongiardini, et al. 2008), and also enhances attachment to non-cognate host plants, soybean and alfalfa (Mongiardini, et al. 2008). Increased expression of *rap* genes also enhances the root attachment of *Rhizobium leguminosarum* bv. *viciae* to pea (Frederix, et al. 2014).

Secondary attachment in *Agrobacterium*

A polysaccharide-containing adhesive structure known as UPP mediates secondary attachment in *A. tumefaciens* (Fig. 2) (Heindl, et al. 2007). UPP localizes to the cell poles of *Agrobacterium*, and facilitates an irreversible and polar attachment that has low specificity and is utilized in binding to both biotic and abiotic surfaces (Matthyssee 2014; Tomlinson and Fuqua 2009). UPP is only observed upon surface contact (Berne, et al. 2015), and it has recently been proposed that UPP secretion is stimulated by elevated intracellular c-di-GMP (Xu, et al. 2013). UPP is a very closely related polysaccharide to glucomannan of *R. leguminosarum* (Laus, et al. 2006). N-acetyl glucosamine has been identified as one of the components of UPP; polarly attached *A. tumefaciens* cells stain with fluorescent wheat germ agglutinin that specifically binds N-acetyl glucosamine residues in polysaccharides (Tomlinson and Fuqua 2009). N-acetyl glucosamine is a monosaccharide derivative of glucose, and this agrees with carbohydrate analysis of glucomannan showing it to consist of almost exclusively glucose and mannose (Laus, et al. 2006). There are likely additional sugars besides N-acetyl glucosamine, and these remain to be defined.

The key difference between the UPP and glucomannan is the irreversible and reversible attachment they respectively facilitate. However, both associations do provide sites for the

attachment and aggregation of increasing numbers of bacteria, and this is facilitated by cellulose fibrillation (Heindl, et al. 2007). The cellulose synthase gene (*celA*) of *A. tumefaciens* shares a high degree of homology with the cellulose synthase of rhizobia and other alpha-proteobacteria (Matthyssee 2014). *A. tumefaciens* and *Rhizobium* share a common mechanism of cellulose synthesis that produces microfibrils scattering from multiple points over the cell surface (Matthyssee 2014). Cellulose fibrils binding tightly to each other, and result of this is the formation of bacterial aggregates on the plant surface. The extent of similarities between the attachment of *Rhizobium* and *Agrobacterium* is unsurprising considering their close phylogenetic relationship. It is characteristic of cellulose production in *A. tumefaciens* that bacteria in the cellulose-bound aggregates are 'tangled' in random orientations (Matthyssee 2014). Fibril formation helps strengthen attachment but is not essential, cellulose synthesis deficient mutants demonstrate weakened but still functional binding to plant surfaces (Matthysse 1983; Matthysse and McMahan 1998). The synthesis of cellulose and UPP are believed to be integrated through c-di-GMP regulation (Matthyssee 2014), and both play prominent roles in the secondary attachment phase of *A. tumefaciens*.

Agrobacterium strains defective in UPP synthesis are no longer able to bind in the prominently polar wild-type fashion (Matthyssee 2014). Although a UPP deficient mutant can still infect a wide range of plants (Tomlinson and Fuqua 2009), the ability to attach is much reduced (Matthyssee 2014). Further investigation into the UPP deficient mutants shows they are removed from the plant surface by water washing at time points where the wild-type is bound irreversibly (Lippincott and Lippincott 1967; Sykes and Matthysse 1986). Never the less, an increased incubation time with the root surface allows secondary attachment and extracellular fibril production to later proceed (Lippincott and Lippincott 1967; Matthysse 1983; Neff and Binns 1985), and this points to the presence of an alternative secondary attachment mechanism that allows attachment in a non-polar and UPP-independent manner. Factors involved in UPP-independent attachment remain unclear, but it is evident that the binding of UPP and synthesis of extracellular fibrils, such as cellulose, constitutes the major mechanism of secondary attachment in *Agrobacterium* species. Separate attachment factors proposed include other exo- and capsular polysaccharides (Reuhs, et al. 1997), other elements on the Ti plasmid, '*att*' gene proteins (Matthysse and McMahan 1998) and proteinaceous fimbrae (Matthyssee 2014; Penalver, et al. 1996).

Secondary attachment in *Azospirillum*

Secondary attachment in *Azospirillum* is similarly characterized by bacterial aggregates forming on the root surface and associated with the biosynthesis of a polysacchardidic fibrillar material (Fig. 2) (Jofré, et al. 2004). The outer surface of *Azospirillum* is composed of two major types of polysaccharide; CPS, a dense capsular polysaccharide that binds tightly to the cells, and EPS, a lighter form that extends out from the CPS (Mora, et al. 2008). Secondary attachment factors in *Azospirillum* include a combination of EPS and CPS, particularly arabinose-rich variants, as well as LPS, outer membrane proteins (OMPs) and

outer membrane lectins (OMLs) (De Troch and Vanderleyden 1996; Del Gallo and Fendrik 1994; Michiels, et al. 1991).

The EPS of *A. brasilense* is proposed to function in cell-to-cell adhesion by acting as either a non-specific flocculant or through forming specific interactions with components of neighbouring bacterial cell envelopes (Burdman, et al. 1998). EPS synthesis is induced as part of secondary attachment, and analysis of cell aggregation ability and EPS production in different *A. brasilense* strains shows EPS concentration and composition directly influences the extent of cell aggregation (Burdman, et al. 2000). Of the monosaccharide components of EPS, L-arabinose plays an especially prominent role (Burdman, et al. 2000). Arabinose is a component of both EPS and CPS, and the concentration of arabinose in both these cell surface components has a positive correlation with the extent of cell attachment and aggregation (Burdman, et al. 2000).

LPS is a significant structural component of the outer membrane in Gram-negative bacteria, and is exposed on the cell surface in *Azospirillum* (Schloter, et al. 1994). LPS is a tripartite molecule consisting of a lipid joined to a polysaccharide 'O-antigen' component by core oligosaccharides. The O-antigen is generally a large and very repetitive polysaccharide domain. Correct structure of these is important for secondary attachment in *Azospirillum* (Balsanelli, et al. 2013). An LPS mutant in both *A. brasilense* and *Heraspirillum seropedicae* with an altered core structure has impaired attachment to maize roots and diminished root colonization (Balsanelli, et al. 2013; Jofré, et al. 2004). The addition of N-acetyl glucosamine inhibits *Azospirillum* attachment to maize roots, and the N-acetyl glucosamine residues of the LPS O-antigen are proposed to directly bind maize root lectins (Balsanelli, et al. 2013).

OMPs and OMLs also contribute to the secondary attachment phase in *Azospirillum* (Burdman, et al. 1999). As previously discussed, constitutively present OMPs are believed to facilitate both primary attachment and cell aggregation (Burdman, et al. 2001). An OML has recently been identified in *A. brasilense* that specifically recognizes and binds to bacterial EPS (Mora, et al. 2008). This 67kDa OML is believed to mediate adhesion between attaching azospirilla cells through EPS bridges (Mora, et al. 2008), and may provide an early step in the formation of larger aggregates on the root surface. Immobilised EPS used in affinity column experiments demonstrates both high affinity and specificity in the binding between EPS and this OML, and also suggests that the lectin targets a complex oligosaccharide structure as opposed to specific single monosaccharides (Mora, et al. 2008).

Secondary attachment in *Pseudomonas*

There is considerable variety in the transition between primary and secondary attachment across pseudomonads. These differences may stem from differences in the nature of the plant-microbe interaction and differences in the surface chemistry and saturation levels of plant

species rooting systems. Firstly, there are differences in the root colonization phenotype. *P. putida* establishes stable biofilm-structures on the root surface, and these reach a maximal size relative to the root after which any further growth is subsequently coupled with growth of the root (Sauer and Camper 2001). In comparison, *P. fluorescens* discontinuously colonizes the root surface and develops as a smaller biofilms along epidermal fissures (Bloemberg and Lugtenberg 2004; Bloemberg, et al. 2000), and finally, *P. syringae* is observed to form dense, merging colonies attached to the surface (Bais, et al. 2004).

An attachment model is proposed in *P. putida* in which two large adhesion proteins (LapF and LapA) act in an orchestrated sequence to facilitate the initiation of secondary attachment and then microcolony formation (Martínez‐Gil, et al. 2010). LapA is believed to drive a transition from reversible, polar attachment to irreversible attachment on the root surface. LapF production is then initiated during these early surface interactions, and the secreted protein mediates cell-cell interactions and is required to drive the transition from singularly attached cells to microcolony assembly. Indeed, LapF deficient mutants have competition defects in the rhizosphere and cannot advance beyond single cell attachments to form aggregated colonies (Fuqua 2010; Martínez‐Gil, et al. 2010). LapF and LapA share commonalities in adhesive-properties, size, secretion, cell-surface localization and both contain expansive repeat domains, but, however, do not possess any substantial sequence identity (Fuqua 2010). LapA is conserved in both *P. putida* and *P. fluorescens* (Yamamoto, et al. 2000), although differing in size between the two species (Fuqua 2010), and it is absent in pathogenic *P. syringae* (Hinsa, et al. 2003). The adhesion domain of LapA is similar to the rhizobium-adhering domain of Rap and cadherin proteins in *Rhizobium* species (Ramey, et al. 2004).

Alongside this model of cell surface protein driven attachment, extracellular fibril production, in particular cellulose, is identified as contributing to this secondary attachment phase in *Pseudomonas* species (Fig. 2) (Cannon and Anderson 1991; Matthyssee 2014; Spiers, et al. 2003). In contrast to *Rhizobium* and *Agrobacterium*, cellulose fibrils in *P. fluorescens* are synthesized as a linear array of sites localized to a single side of the cell to form a sheet (Cannon and Anderson 1991; Matthyssee 2014; Spiers, et al. 2003). The geometry of cellulose production is an important factor in influencing the phenotype of bacterial aggregation, and the differences in production geometry are observed to correlate with coding differences in *celB* (Matthyssee 2014).

Secondary attachment in *Salmonella*

Secondary attachment is comparatively poorly characterised in *Salmonella*. Whilst attachment to plant leaves is reasonably characterised, it is unknown the extent to which root attachment is similar. Cellulose is involved in facilitating secondary attachment to roots (Barak, et al. 2007; Charkowski, et al. 2002), similarly to the secondary attachment mechanisms observed in *Rhizobium, Agrobacterium, Azospirillum* and *Pseudomonas*. The role of cellulose in

Salmonella root colonization is probably likewise in cell-cell interactions and aggregation (Barak, et al. 2007). A recent transposon mutant screen carried out to identify attachment factors in *S. enteric* suggests a number of potential secondary attachment candidates (Fig. 2) (Barak, et al. 2005). This includes a cell surface aggregate fimbria nucleator (encoded by *agfB*) that may be important in cell aggregation on the root surface and the RpoS, which has a number of regulatory roles, the most relevant of these being in cellulose production. Considering what is known for other rhizobacteria, particularly the more closely related pseudomonads, it is likely we are yet to identify a number of other cell surface proteins and polysaccharides that are involved in *Salmonella* secondary attachment.

Drawing together a common biphasic model of root attachment

In terms of drawing together a common biphasic model of root attachment (Fig. 3), there is the weak and reversible primary attachment phase, followed by the stronger and more specific secondary attachment phase, that is generally accompanied by extracellular fibril synthesis and the formation of bacterial aggregates on the root. Microbes typically use surface appendages such as flagella or pili to migrate towards the root (Fig. 3). Initial primary attachment is dictated by weak electrostatic and physiochemical binding forces developing between surface molecules of the root and bacterial cell envelope (Berne, et al. 2015). However, these charge-based interactions are only really important on a very early timescale, and significantly stronger associations develop through microbe-specific primary attachment factors, such as surface polysaccharides, proteins, OMPs, flagella and pili (Fig. 1). EPS is an example of a cell surface constituent that can influence both the primary and secondary attachment phases across different bacterial species. The type of EPS present will modify cell surface electrostatic, hydrophobic and steric properties, all of which influence the initial primary interactions, and the polysaccharide is also able to help facilitate cell aggregation and irreversible binding to the surface.

Primary attachment is mediated by constitutively present cell surface components, whose protrusive domains form bonds with components of the root cell surface (Fig. 1). For example, glucomannan of *R. leguminosarum* binds to lectin receptors on pea and vetch roots (Laus, et al. 2006), and the 47.7 kDa major OMP of *A. brasilense* facilitates initial adsorption onto wheat roots (Burdman, et al. 1999). Secondary attachment is commonly facilitated by the induced biosynthesis, secretion or exposure of specific secondary attachment factors and fibrillar material (Fig. 2). For example, this is seen with the stimulation of UPP secretion upon surface contact in *Agrobacterium* (Berne, et al. 2015), and EPS synthesis is induced as part of the secondary attachment mechanism in *A. brasilense* (Burdman, et al. 2000). However, this induction is not always the case, and constitutively present cell surface components, such as OMPs, OMLs and EPS, also function in mediating strong secondary attachment associations and cell aggregation. Cellulose is a universal secondary attachment factor for all microbes discussed within this review, and clearly provides important agglutinating activity in plant root colonisation.

In some species, regulation of the transition from primary to secondary attachment is very well defined, such as the orchestrated sequence in which LapA and LapF function in *P. putida* (Martínez‐Gil, et al. 2010). This understanding of LapA and LapF is one of the most significant recent advances in plant-microbe attachment research. In other species, it is less evident how secondary attachment factors are induced, although in the case of *Agrobacterium*, surface contact and c-di-GMP signalling certainly appear to play a role (Matthyssee 2014). It seems logical in all cases that either secondary attachment associations will form through constitutively present cell surface factors, or surface contact as either 'sensed' by the binding of primary attachment factors or through another cell surface component will trigger signalling to induce biosynthesis, secretion or exposure of specific secondary attachment factors.

Alongside a role in motility, flagella and pili can facilitate adhesion, and this has been identified independently in numerous species of *Agrobacterium, Azospirillum, Pseudomonas* and *Salmonella*. Flagella and pili mutants are commonly used to investigate a role in attachment, but in this research it is not always entirely clear whether the structures have a direct physical role in attachment themselves, or if they are required for moving towards the roots. However, many studies are able to demonstrate a direct physical role in attachment, such as with the purified polar flagella of *A. brasilense* binding directly to the wheat root surface (Croes, et al. 1993). This role could perhaps be better distinguished by examining motor mutants (*mot*) that have normal flagella that cannot rotate and so are unable to swim, but should still possess any adhesive domain functions. Although it has not yet been shown for all rhizobacteria that flagella and pili possess this duel role and function directly in root attachment, the fact that they function in this adhesive manner in a wide range of other strains suggests it is possible it is a universal trait.

Flagella and pili are known to play a direct adhesive role in attachment to both abiotic surfaces and in animal colonisation across wide range of gram-negative and gram-positive bacteria (Berne, et al. 2015). It has been well-characterised for attachment to abiotic surfaces that pili are commonly involved in the lifestyle switch between motile and attached, and this has been studied in depth with the chaperone-usher pathway (CUP) pili of *Escherichia coli* and the tight adhesion (Tad) pili of *Caulobacter crescentus* (Entcheva-Dimitrov and Spormann 2004; Pratt and Kolter 1998). The motility role of flagella and pili in this review is focused on the "swimming" of cells towards the roots, but these appendages can also mediate a coordinated "swarming" of bacterial populations on solid surfaces (Jarrell and McBride 2008). More recently, the specific role of surface swarming in root colonisation has began to be investigated (Dietel, et al. 2013; Verstraeten, et al. 2008), and it appears both the swimming and swarming functions of these appendages play important roles in facilitating attachment and colonisation. Swarming is likely more important in plant-microbe interactions involving biofilm formation on the roots as opposed to interactions involving an endophytic or infection based colonisation.

Expansion across other species

The five bacterial genera discussed in this review spread across a range of agriculturally important interactions for species whose root attachment mechanisms have received some of the most significant research attention to date. As previously mentioned, the vast majority of root attachment research has thus far been carried out in plant-microbe interactions involving Gram-negative bacteria (Bogino, et al. 2013; de Jesus Sousa and Olivares 2016; Rodríguez-Navarro, et al. 2007), and the root attachment mechanisms of Gram-positive species has only more recently become a bigger focus of attention (Habib, et al. 2017; Viaene, et al. 2016). Abiotic biofilm formation has generally been better characterised in species of plant-interacting Gram-positive bacteria, and is shown to follow the same biphasic mechanism of the initial reversible attachment of single cells, followed by irreversible attachment of multiple cells at the site of initial adhesion (Beauregard, et al. 2013; Habib, et al. 2017; Viaene, et al. 2016). However, it is unknown how similar this is to an attachment mechanism that occurs *in planta*. Many processes in attachment and colonisation seem to be conserved among bacteria (Poole 2017). For instance, numerous species of both Gram-negative and Gram-positive bacteria have been identified with requirements for EPS and cellulose in attaching to plants (Bogino, et al. 2013; Poole 2017), and pili in Gram-positive bacteria have also been suggested to possess duel roles in both motility and attachment (Mandlik, et al. 2008).

Bacillus subtilis has long been known to provide plant growth promoting effects through association with plants in the rhizosphere, and only recently evidence has shown that there is also the formation of root-associated biofilms in response to plant polysaccharide signals, which also serve as a source of sugars for matrix EPS synthesis (Beauregard, et al. 2013; Habib, et al. 2017). The biofilm matrix of *B. subtilis* consists of EPS and protein fibres (TasA and TapA), in which TasA is believed to play a similar structural role as to the secondary attachment lectins in *A. brasilense* that form EPS bridges between cells. *Streptomyces* are similarly renowned for their plant growth effects; they are commonly found inhabiting the soil and rhizosphere and are able to produce a variety of bioactive secondary metabolites (Viaene, et al. 2016). Both classical and new techniques convincingly show that *Streptomyces* attaches to roots, but these attachment strategies are poorly understood (Viaene, et al. 2016). Attachment to abiotic hydrophobic surfaces can be mediated by extracellular fimbriae constructed of chaplin protein monomers assembling along cellulose fibrils (De Jong, et al. 2009). Cellulose acts as a scaffold for the bundling of these protein monomers and mutational studies indicate the presence of a cellulose synthase gene to be involved in attachment (De Jong, et al. 2009). Cellulose has been identified as an important attachment component across the various other rhizobacteria discussed in this review, and it can therefore be hypothesised that cellulose may similarly be required for *Streptomyces* attachment in the biotic context of plant roots. The root attachment mechanism of *Streptomyces* needs to be better understood before fit to the common biphasic mechanism outlined in this review can be confidently assessed.

For many species, research into root attachment does not yet extend past *in silico* genome analysis. For instance, a recent analysis of *Bacillus aryabhattai* AB211 was able to suggest that the genome does indeed encode the necessary arsenal required for adhesion to root surfaces (Bhattacharyya, et al. 2017). The genome possesses a repertoire of EPS biosynthesis and cell surface export genes, which may aid multicellular irreversible adhesion as part of a secondary attachment mechanism, but this remains to be validated *in vivo*. *Escherichia coli* is another microbe whose root attachment mechanism has received research attention, and similarly to *Salmonella*, it has been implicated in numerous food poisoning outbreaks associated with the consumption of contaminated food (Martínez-Vaz, et al. 2014; Wiedemann, et al. 2015). A microarray analysis study looking at the interaction between *Escherichia coli* and lettuce roots found that genes involved in curli formation and biofilm modulation were important for attachment (Hou, et al. 2012). Curli are extracellular proteinaceous extrusions involved in mediating cell adhesion in both *E. coli* and *Salmonella* (Barnhart and Chapman 2006), and biofilm modulators, extracellular CPS, curli and flagella have all been suggested to be involved in the molecular mechanism by which *E. coli* colonises plant roots (Martínez-Vaz, et al. 2014; Seo 2013). Parallels have been drawn between the attachment mechanisms utilised by *Salmonella* and *E. coli*, and similarly to *Salmonella*, a primary attachment phase driven by extracellular structures, specifically curli/fimbriae and flagella, has also been suggested in *E. coli* (Martínez-Vaz, et al. 2014). Current research strongly suggests that genes involved in biofilm formation are important for the root attachment of *E. coli* (Hou, Martínez-Vaz, et al. 2014), however, the formation of mature biofilms by *E. coli* in natural plant environments has not yet been documented (Martínez-Vaz, et al. 2014). The utilisation of these biofilm modulator genes would most likely occur as part of a secondary attachment mechanism, although it remains possible that different mechanisms using similar genetic responses are being utilised in plant colonisation.

Concluding remarks

Attachment to plant roots is a universally important step in plant colonization across symbiotic, pathogenic and plant growth promoting interactions. A common biphasic mechanism of root attachment consisting of a primary and a secondary attachment phase is observed in agriculturally important rhizobacteria species. Despite the fact that attachment capabilities are a central factor in determining microbial competitiveness in root colonization (Rodríguez-Navarro, et al. 2007), much remains unclear about the molecular mechanisms governing root attachment, particularly in Gram-positive bacterial species (Bogino, et al. 2013; Viaene, et al. 2016). It is evident that our knowledge of attachment factors is incomplete, for instance, what mediates UPP-independent attachment in *Agrobacterium*? Which factors are involved in primary and secondary attachment in *Salmonella*? Is there a rhicadhesin encoded in *Rhizobium* and *Agrobacterium* genomes? The plant-microbe interactions of *Rhizobium* and *Agrobacterium* have been studied for decades, but for *Salmonella*, root attachment is only recently a focus of attention. Within these plant-microbe interactions, it has proven difficult to identify attachment factors, quantify their role within an *in vivo* system and piece together multifaceted molecular mechanisms. The complex networks that underpin key biological processes are often difficult to tease apart, and for studying root

attachment, there are also the technical difficulties in protein purification, problems with the accuracy of targeted mutational studies and limitations in the power of attachment assays.

Looking to the future, a number of recent technologies provide promising avenues to improve our understanding of this important biological mechanism. These include transposon insertion mutation studies to carry out high-throughput whole genome screening approaches (Perry 2014; Wheatley, et al. 2017), Tracking Root Interaction Systems (TRIS) to observe real-time attachment dynamics with high resolution microscopy (Massalha, et al. 2017), lux-based reporter systems to measure attachment in a medium-throughput fashion (Pini, et al. 2017), and *omics* technologies to aid the structural determination of bacterial surface molecules (Rodríguez-Navarro, et al. 2007). One limitation is that most commonly used measurements of attachment involve microscopy and colony counting, both of which are labour intensive, time intensive and reasonably susceptible to human error. A quantitative real-time PCR assay to measure *A. tumefaciens* attaching has recently been developed (Petrovicheva, et al. 2017). This measure lends itself to various applications, including the more high-throughput ability to test effects of mutations on bacterial adhesion molecules and to compare attachment across numerous plant species. Lux-based reporter systems provide significant advantages in terms of ease and cost-efficiency. They allow spatiotemporal mapping of bacteria attaching to the root, and can be used to give a striking visualisation of the phases of attachment (Pini, et al. 2017). From drawing together a common biphasic model of root attachment, it is clear that the knowledge acquired in a particular species can provide insight into a vast array of other plant-microbe interactions. Hopefully utilising these new investigative technologies will enable a more complete picture of the molecular mechanisms governing root attachment to be acquired, and from this, a better understanding of how to manipulate these plant-microbe interactions for agricultural benefit.

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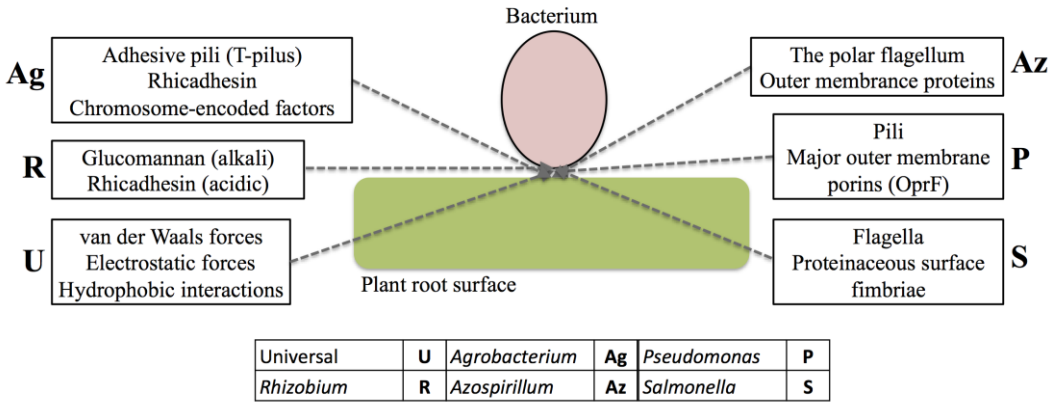
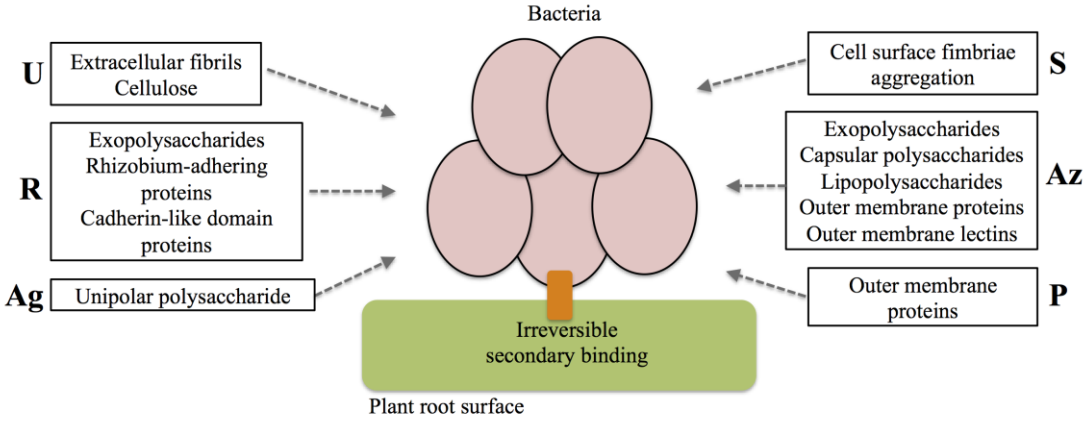


Figure 1. Molecular mechanisms of primary attachment allowing the weak and reversible binding of a single cell to the root. Universal binding forces and microbe-specific attachment factors are indicated: U; Universal, R; *Rhizobium*, Ag; *Agrobacterium*, Az; *Azospirillum*, P; *Pseudomonas*, S; *Salmonella*.



Universal	**U**	*Agrobacterium*	**Ag**	*Pseudomonas*	**P**
Rhizobium	**R**	*Azospirillum*	**Az**	*Salmonella*	**S**

Figure 2. Molecular mechanisms of secondary attachment allowing stronger and irreversible binding to the root, and characterized by the synthesis of cellulose and other extracellular fibrils aiding the accumulation of bacterial aggregates. Universal attachment factors and microbe-specific attachment factors are indicated: U; Universal, R; *Rhizobium*, Ag; *Agrobacterium*, Az; *Azospirillum*, P; *Pseudomonas*, S; *Salmonella*.

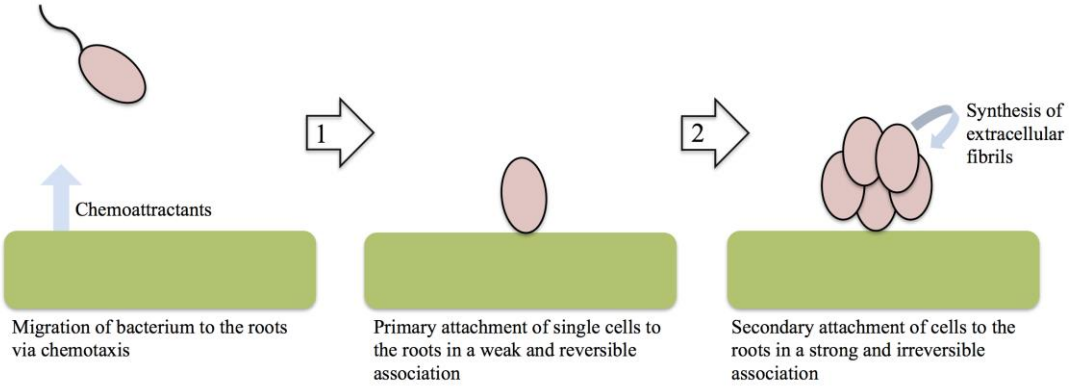


Figure 3. The biphasic model of root attachment. Bacteria migrate towards the roots via chemotaxis in response to root exuded chemoattractants. Step 1 of the biphasic model is the primary attachment phase, where a bacterium attaches to the root in a weak and reversible manner. Root association is initially dictated by physiochemical and electrostatic attractions between the surface molecules of the root and bacterial cell envelope. Stronger microbe-specific primary attachment factors (Fig. 1) may then bind to the root surface and will dictate the overall mechanics of primary attachment. Step 2 of the biphasic model is the secondary attachment phase, where strong and irreversible binding between the bacterium and the root surface occurs. There is the synthesis of extracellular fibrils, cellulose and microbe-specific secondary attachment factors (Fig. 2) and the formation of bacterial aggregates.