Chapter 116

The Rhizosphere as a Reservoir for Opportunistic Human Pathogenic Bacteria

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116.1 INTRODUCTION

The interface between soil and plant roots—the rhizosphere—is, because of root exudates and the resulting high nutrient content, a unique microenvironment in terrestrial ecosystems (Sørensen, 1997; Raaijmakers et al., 2009). Cultivation-independent methods on the basis of DNA/RNA, such as microbial fingerprinting techniques, fluorescence *in situ* hybridization, and pyrosequencing, have given interesting insights into the structure of rhizosphere-associated bacterial communities (reviewed in Smalla 2004; Hartmann et al., 2009; Mendes et al., 2011). But what do we know about the functions of plant-associated bacteria? Firstly, bacteria play a role in plant growth. They can support nutrient uptake, enhance the availability of phosphorous, and produce a broad range of phytohormones (Costacurta and Vanderleyden, 1995). An interesting phenomenon is the enhancement of stress tolerance by lowering the ethylene level (Glick, 1998). Another important function is the involvement of plant-associated bacteria in pathogen defense. Many pathogens attack plants, especially fungi, oomycetes, and nematodes, and cause yield losses of more than 30% worldwide. While resistance against leaf pathogens is often encoded in the plant genome, it is difficult to find resistance genes against soil-borne pathogens. Cook et al. (1995) suggest that antagonistic rhizobacteria fulfill this function. Interestingly, besides direct antagonism, plant-associated bacteria can induce a systemic response in the plant, resulting in the activation of plant defense mechanisms (Pieterse et al., 2003). Besides the general microbe-associated molecular patterns (MAMPs) for recognition of microbes by the plants' innate immune system, more recently bacterial quorum sensing signaling molecules have been identified to induce systemic resistance against biotrophic plant pathogens (Schuhegger et al., 2006; Schikora et al., 2011; Schenk et al., 2012).

Studying plant-associated bacteria and their structure and functions is important not only to understand their ecological role and the interaction with plants and plant pathogens but also for any biotechnological application. In biotechnology, plant-associated bacteria can be applied directly for biological control of plant pathogens as biological control agents (BCAs), for growth promotion as plant growth-promoting rhizobacteria (PGPR), and enhancement of stress tolerance as biofertilizers and phytostimulators or as rhizoremediators (Whipps, 2001; Berg, 2009). To avoid any risk of the application of these microbial inoculants for human health, it is important to understand the mode of interaction with eukaryotic

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hosts (Berg et al., 2010). On the other side, knowledge of the pathogenic potential of rhizobacteria can help to understand and discover newly emerging pathogens.

116.2 THE RHIZOSPHERE AS RESERVOIR FOR OPPORTUNISTIC HUMAN PATHOGENIC BACTERIA

During the last few years, it has been shown that plants, especially in the rhizosphere, can harbor not only beneficial bacteria but also those that can potentially cause diseases in humans (reviewed in Berg et al., 2005). Opportunistic pathogens can only cause diseases in patients with a strong predisposition to illness (Parke and Gurian-Sherman, 2001; Steinkamp et al., 2005). This group of bacteria cause the majority of bacterial infections associated with significant case/fatality ratios in susceptible patients in Europe and Northern America (Vincent et al., 1995) and their importance is still increasing worldwide.

Many plant-associated genera, including *Burkholderia, Enterobacter*, *Cronobacter*, *Herbaspirillum*, *Azospirillum*, *Ochrobactrum*, *Pantoaea*, *Pseudomonas*, *Ralstonia*, *Serratia*, *Staphylococcus*, and *Stenotrophomonas* contain root-associated and endophytic bacteria that engage in bivalent interactions with plant and human hosts. Several strains of these genera show plant growth promoting as well as excellent antagonistic properties against plant pathogens and have therefore been applied as BCAs or PGPRs (Whipps, 2001). However, many strains also successfully colonize human organs and tissues and thus cause diseases as shown for members of the *Burkholderia cepacia* complex (Govan et al., 2000; Parke and Gurian-Sherman, 2001). This underlines the importance of thorough risk assessment studies before registration of microbial inoculants.

116.3 FACTORS INVOLVED IN EUKARYOTE–MICROBE INTERACTION

Rhizosphere-associated bacteria with antagonistic activity against eukaryotes are able to interact with their hosts using various mechanisms. These mechanisms include (i) recognition and adherence, (ii) inhibition of pathogens by antibiotics, toxins, and biosurfactants (antibiosis), (iii) competition for colonization sites and nutrients, (iv) competition for minerals, for example, for iron through production of siderophores or efficient siderophore-uptake systems, (v) degradation of pathogenicity factors of the pathogen such as toxins, and (vi) parasitism that may involve production of extracellular, cell wall-degrading enzymes such as chitinases and β-1,3 glucanases (Lugtenberg and Kamilova, 2009). Other factors that contribute to rhizosphere fitness include the ability to use seed and root exudates as carbon sources or, more generally, ecological and nutritional versatility. Steps of pathogenesis are similar and include invasion, colonization and growth, and several strategies to establish virulence (the relative ability of a pathogen to cause disease in the host). Each of the factors involved has been referred to as *virulence factor* or *pathogenicity factor* (Dobrindt et al., 2004). Many mechanisms involved in the interaction between antagonistic plant-associated bacteria and their host plants are similar to those responsible for pathogenicity of bacteria (Rahme et al., 1995; Cao et al., 2001). Extensive invasion and endophytic colonization of plants have been demonstrated for food-borne pathogens such as *Salmonella enterica* pv. Typhimurium in barley (Kutter et al., 2006) and for the severe human pathogen *Burkholderia pseudomallei* in diverse plants in Northern Australia (Kaestli et al., 2011). In addition, for the food-borne pathogen *Cronobacter* (former *Enterobacter*) *sakazakii* that is associated with cases of meningitis, necrotizing enterocolitis and sepsis in neonates and immunocompromised infants, endophytic colonization of roots by clinical isolates has been shown (Schmid et al., 2009) (Fig. 116.1). In addition, isolates of *C. sakazakii* have been obtained from the rhizosphere of *Salicornia* and other plants (Jha et al., 2012; Schmid et al., 2009).

Several studies have provided evidence that similar or even identical functions are responsible for beneficial interactions with plants and virulence in humans. For example, the involvement of siderophore-uptake systems or extracellular enzymes is common to both beneficial bacteria and human pathogens (Tan et al., 1999). Dörr et al. (1998) reported that type IV pili of the plant-associated *Azoarcus* sp. BH72 are responsible for the adhesion on plant and fungal cells. Furthermore, the amino acid sequence of the pilus showed a high similarity to pili of the human-associated strains of *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*. Type III secretion systems are responsible for the introduction of effectors into eukaryotic host cells; they have been discovered for pathogenic bacteria as well as plant-associated bacteria with beneficial effects on host plants (Preston et al., 2001).

In a study published by Alonso et al. (1999), it was shown that clinical and environmental isolates of *P. aeruginosa*, which is the major causal agent for morbidity and mortality of patients with cystic fibrosis, share several phenotypic traits with respect to both virulence and environmental properties. Several studies support the view that the environmental strains are indistinguishable from those arising from clinical sources in terms of genotypic, taxonomic, or metabolic properties (Kiewitz

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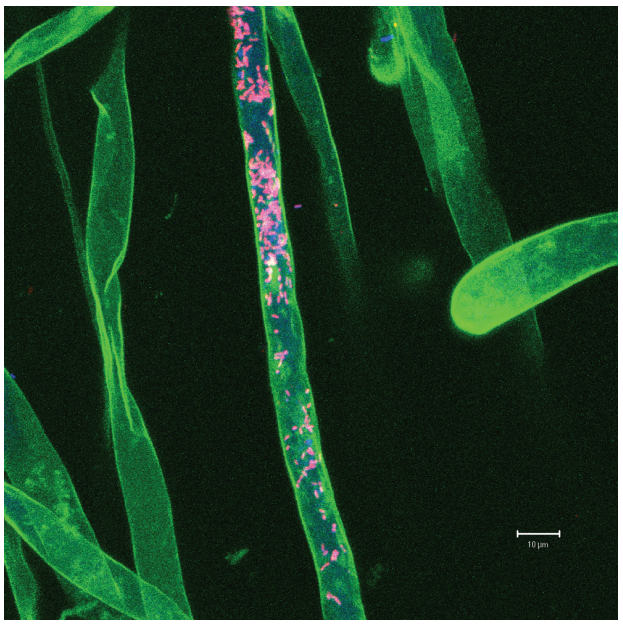


Figure 116.1 Root colonization of axenically grown tomato plants inoculated with the clinical isolate *Cronobacter sakazakii* ATCC 29544^T and hybridization with the oligonucleotide probes EUB-338-I, -II, -III-Cy5 (blue), specific for the domain bacteria and GAM-42a-Cy3 (red), specific for the γ-subclass of proteobacteria using the fluorescence *in situ* hybridization protocol (Amann et al., 1995). The composed rgb-image results in magenta color for bacteria, indicating colabeling by EUB-338-I, -II, -III-Cy5, and GAM-42a-Cy3. The remaining third color channel (green) was used to visualize autofluorescence and thus the structure of roots.

and Tümmler, 2000; Finnan et al., 2004; Morales et al., 2004). In addition, differences between environmental strains and those that cause infections might rather be due to the regulation of genes, than their mere presence or absence (Parke and Gurian-Sherman, 2001). In this regard, similar studies with focus on *P. aeruginosa, Stenotrophomonas maltophilia* (reviewed in Ryan et al., 2009), and *B. cepacia* (Parke and Gurian-Sherman, 2001) have been published. Nevertheless, antagonism studies and biocontrol effects have been reported for all mentioned species, and one product derived from *B. (ceno)cepacia* has been on the market (Hebbar et al., 1998; Nakayama et al., 1999; Dunne et al., 2000, Govan et al., 2000). These species are common inhabitants of the rhizosphere; yet because of their medical relevance, they are grouped into risk group 2 in public databases, for example, of the DSMZ-German Collection of Microorganisms and Cell Cultures (www.dsmz.de), and should be excluded from direct biotechnological applications.

An important mechanism by which harmless bacteria can behave as pathogens is the change of host or host niche, upon which their virulence potential is frequently released to its full extent. This mechanism is clearly relevant for opportunistic pathogens from plant-associated habitats. In addition, other mechanisms such as structural changes of the bacterial chromosome due to gene acquisition and loss, recombination, and mutations can lead to bacterial pathogenicity (for a review, see Hacker et al., 2003). Genes responsible for pathogenicity or fitness of bacteria often occur as genomic islands, which are blocks of DNA with signatures of mobile genetic elements (Hacker and Carniel, 2001). They are called *fitness islands* or *pathogenicity islands* according to their function.

Rhizosphere-associated bacteria with a high capacity for biocontrol can be potentially dangerous for human health. Therefore, it is important to understand the mode of action and specific properties of the BCA. It is well known that antagonistic properties and underlying mechanisms are highly strain specific (Berg et al., 2002), but identification of bacteria is based mainly on 16S-rDNA sequencing. However, based on the sequence information of the ribosomal RNA, which is a central and well-conserved housekeeping gene, it is impossible to draw conclusions about potential pathogenicity: neutral bacterial strains can be dangerous because of pathogenicity islands or pathogenic bacteria can be harmless because of the absence of any pathogenicity factor.

116.4 *STENOTROPHOMONAS*: AN AMBIVALENT GLOBAL PLAYER FROM THE RHIZOSPHERE

Bacteria of the genus *Stenotrophomonas* are of increasing biotechnological interest because of their ubiquitous occurrence and versatility (Ryan et al., 2009). Their plant growth-promoting properties and their antagonistic behavior against soil-borne plant pathogens are well documented, but development for commercial application of *S. maltophilia*, the most intensively studied species, has been hampered by its potential as opportunistic human pathogen in immune-suppressed patients (reviewed in Ryan et al., 2009). Within a broad range of isolates of environmental and clinical origin, classified at the time as *S. maltophilia*, a distinguished genomovar consisting only of environmental isolates could be separated (Minkwitz and Berg, 2001), and further characterization led to their description as a separate species, *Stenotrophomonas rhizophila* (Wolf et al., 2002). In contrast to *S. maltophilia*, *S. rhizophila* does not have human pathogenic traits (Ribbeck-Busch et al., 2005; Hagemann et al., 2006), has a lower temperature optimum than *S. maltophilia* (Wolf et al., 2002), and is therefore safe to apply in biotechnological approaches. In addition, the synthesis of an additional osmolyte, glucosyl glycerol, confers a greater degree of salt resistance *in vitro* (Hagemann et al.,

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2008) and makes it an ideal candidate for applications under saline soil conditions. *In vitro*, isolates of this species produce fungal cell wall-degrading enzymes, siderophores (Minkwitz and Berg, 2001), and volatile antifungal compounds (Kai et al., 2007); antifungal activity against the soil-borne plant pathogens has also been demonstrated (Minkwitz and Berg, 2001). Moreover, production of the plant growth hormone indolacetic acid (IAA) and direct growth promotion of strawberry plants *in vitro* have been shown (Suckstorff and Berg, 2003). Furthermore, growth of a wide variety of crops was strongly promoted in the saline soil of Uzbekistan by *S. rhizophila* strain DSM14405^T (=e−p10^T, =P69^T) (Egamberdieva et al., 2011). These studies indicate the potential of *S. rhizophila* to directly promote plant growth as well as to inhibit plant pathogens.

All this information is based on physiological and targeted molecular investigations—but how does this information correlate with results obtained by genome sequencing? Although *S. rhizophila* is grouped in risk group 1 and no human infection has ever been reported till now, genome sequencing of DSM14405T revealed numerous potential virulence factors. For example, the type VI secretion system (T6SS), a bacterial transport system, recently discovered in a number of important human, animal, and plant pathogenic Gram-negative bacteria, has drawn attention of many scientists in the past years because of its role in causing virulence and pathogenesis in the host organisms. Some clinically important pathogenic bacteria that use T6SS for secretion of virulence factors into the cytosol of human cells are as follows: *Burkholderia mallei*, *Yersinia pestis*, *Salmonella typhimurium*, and *Legionella pneumophila* (Purcell and Shuman, 1998; Parsons et al., 2005; Schell et al., 2007). In addition, *P. aeruginosa*, an opportunistic pathogenic but also plant-associated bacterium, possesses T6SS (Mougous et al., 2006). Surprisingly, our genome analysis investigations have revealed that *S. rhizophila* DSM14405^T also possesses a gene cluster with at least 19 genes being directly related to the structure and function of T6SS. Genes known to generate the backbone of T6SS are conserved among bacteria with a functioning type VI secretion system. The most important members are *icmF*, *clpV* encoding the corresponding ATPase, *hcp* encoding the hemolysin coregulated protein, and the *Vgr*-related genes (Bingle et al., 2008). The *S. rhizophila* DSM14405^T T6SS gene cluster possesses homologs to all these genes. Other genes known to belong to T6SS in various other bacteria are also present in the *S. rhizophila* T6SS gene cluster, such as an *impA*-related N-terminal family gene, which has a homolog in *Burkholderia pseudomallei* 7894, the pathogenic agent for melioidosis in humans. Figure 116.2 shows the T6SS gene cluster in *S. rhizophila* DSM14405^T, with the most important components noted above being highlighted. The physiological role of T6SS in *S. rhizophila* as a BCA and plant growth-promoting agent is still unknown, but there is a great chance for its involvement in the secretion of a broad range of effectors and other molecules with importance for bacteria–host plant and bacteria–plant pathogen interactions.

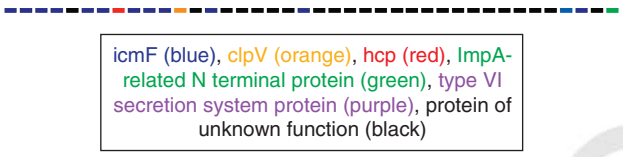


Figure 116.2 Gene organization in the type VI secretion system cluster of *Stenotrophomonas rhizophila* DSM14405^T derived from protein BLAST analysis investigations, the most crucial genes have been highlighted. Some genes with unknown functions in other bacteria could play as strain specific effector molecules or belong to the structural component set of type VI secretion system in *S. rhizophila* DSM14405^T.

Aside from type VI secretion system, the genome analysis investigations have revealed that the genome of *S. rhizophila* DSM14405^T harbors other genes encoding proteins/systems of ambivalent significance with occurrence in both plant-associated and human/animal pathogenic bacteria. For instance, there are pilin synthesis and assembly genes present in the genome of *S. rhizophila*. An example thereof is a gene that is homologous to *pilE* (pilin protein) from *Legionella longbeachae*, a soil bacterium known to be capable of causing legionellosis in humans (Fields et al., 2002). Moreover, *S. rhizophila* possesses a nonhemolytic phospholipase C gene (*plC*), which has homologs in the clinical *S. maltophilia* K279a, as well as other pathogenic bacteria such as *P. aeruginosa* and *B. pseudomallei*. PLCs are virulence factors, which degrade cell membrane phospholipids. In *P. aeruginosa*, they play a role in bacterial survival in the human endothelial cells (Plotkowski and Meirelles, 1997). Another group of virulence factors important for invasion of host cells are bacterial metalloproteases (Justice et al., 2008). The *S. rhizophila* genome harbors a gene encoding a metalloprotease, which has homologs in *S. maltophilia* K279a (Smlt1595), the beneficial plant-associated *S. maltophilia* R551-3 and *P. aeruginosa*. Another important virulence feature of many human pathogenic bacteria is their resistance towards a broad range of antibiotics (Walsh, 2003). The genome of *S. rhizophila* DSM14405^T also contains some antibiotic resistance genes. For instance, it harbors a gene coding for an aminoglycoside phosphotransferase, which is homologous to Smlt0191 from the clinical strain *S. maltophilia* K279a and Stemr_0151 from the beneficial endophytic strain *S. maltophilia* R551-3. *S. maltophilia*, however, shares more strongly the feature of possessing

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antibiotic resistance genes with human pathogenic bacteria, as our genome comparison analyses revealed. There are a number of antibiotic resistance genes and gene clusters present in both *S. maltophilia* R551-3 and K279a strains, which do not exist in the genome of *S. rhizophila*. The gene clusters Smlt4474-76 and Smlt2796-8 from *S. maltophilia* K279a (Stemr_3899-3901 and Stemr_2294, Stemr_2297 from *S. maltophilia* R551-3) encoding RND-type tripartite efflux system and a multidrug/fusaric acid resistance channel, respectively, are examples of specific differences (Ryan et al., 2009).

116.5 DIAZOTROPHIC PLANT-GROWTH-PROMOTING RHIZOBACTERIA

116.5.1 The *Burkholderia* Genus

Diazotrophic bacteria with plant growth promotion and biocontrol activity related to the *B. cepacia* complex are regularly isolated from diverse rhizospheres, especially from the rhizosphere of rice (Coenye and Vandamme, 2003; Jha et al., 2009). *Burkholderia vietnamensis* has been known since long time as a diazotrophic PGPR (Gillis et al. 1995), but it is a member of the *B. cepacia* complex and thus suspected to harbor pathogenicity features and is banned from application (Coenye and Vandamme, 2003). However, plant-associated *Burkholderia* species, such as *B. unamae*, *B. tropica*, and *B. silvatlantica*, have been described more recently, which belong to a separate branch of the genus *Burkholderia* (Caballero-Mellado et al., 2004; Reis et al., 2004; Perin et al., 2006). Some of these plant-associated *Burkholderia* isolates have been derived from nodules of tropical legumes and were demonstrated to be able to induce the formation of nitrogen fixing nodules (e.g., *B. tuberum* and *B. phymatum*) (Mouldin et al., 2000; Vandamme et al., 2002). The plant-associated/symbiotic branch of *Burkholderia* needs more deep investigations concerning the relationship to the *B. cepacia* cluster and if there is also opportunistic pathogenic potential in these endophytic diazotrophs.

116.5.2 The *Herbaspirillum* Genus

Within the genus *Herbaspirillum*, which includes *Herbaspirillum seropedicae* as a typical endophytic plant growth-promoting rhizobacterium, Baldani et al. (1996) described *H. rubrisubalbicans* as a mild pathogen of certain sugar cane varieties and *Herbaspirillum* species 3, which harbored mostly isolates from clinical specimens. In the meantime, new isolates characterized as *H. seropedicae* (e.g., strain 14010) are known to have opportunistic clinical characteristics (Helisson Faoro and Fabio Pedrosa, personal communication). Recent preliminary DNA-sequencing data revealed that strain 14010 lacks the *nif*-genes and type III secretion system but harbors type 3 fimbriae similar to pathogenic *Klebsiella pneumoniae*. Certainly, further studies on the proteomic level are needed to clarify this comparison of rhizospheric and clinical isolates of *H. seropedicae*.

116.5.3 The *Azospirillum* Genus

Within the genus *Azospirillum*, no phylogenetic closely related clinical isolates were known until recently. For several decades, various strains of *Azospirillum* (mostly *Azospirillum brasilense* and *A. lipoferum* strains) have been successfully used as biofertilizers in millions of ha worldwide to enhance growth of wheat, maize, and other crops (Hartmann and Bashan, 2009) without any reported adverse impact on human health. Cohen et al. (2004) published data on the isolation and characterization of *Azospirillum* spp. from the wheat rhizosphere, *Rhizoctonia solani* mycelia, and human skin wounds and reported a very close relationship of these isolates to *Roseomonas*, a genus described in 1993 as being associated with bacteremia and other human infections (Rihs et al., 1993). In particular, a close resemblance to *Roseomonas fauriae* and *Roseomonas* genomospecies 6 was found. This even led Helsel et al. (2006) to suggest that *R. fauriae* (Rihs et al., 1998) should be reclassified as *A. brasilense* (Tarrand et al., 1979). The evidence provided were a series of biochemical tests and molecular phylogenetic data, which apparently could not differentiate between the two taxa. A thorough reexamination of this comparison by Hartmann and coworkers (unpublished results) revealed that the whole sequences of the 16S-rRNA- and 23S-rRNA-genes, the ITS1-region of the rRNA-operon, as well as the *nifH*- and the *rpoB*-genes of *A. brasilense, R. fauriae*, and *Roseomonas* genomospecies 6 are indeed highly similar. Their sequence similarity ranged from 95% (ITS1 region) to 98–99% for the 16S and 23S rRNA genes. However, when the % DNA–DNA relatedness was examined, a value of 12% was found between *R. fauriae* KACC11694^T and *A. brasilense* Sp7^T and 25% between *Roseomonas* genomospecies 6 CCUG 22010 and *A. brasilense* Sp7^T. This clearly demonstrates that these bacteria are obviously separate, possibly even on the genus level, although the ribosomal and other housekeeping genes are very similar. The examination of the fatty acid content and the physiological markers according to BIOLOG Microplate Systems^R also revealed clear differences (Kinzel et al., unpublished); 9–12 (*R. fauriae*) and 5–6 (*Roseomonas* genomospecies 6) differences were found in the biochemical utilization patterns

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of carbon substances in these strains as compared to different *A. brasilense* strains. Therefore, *R. fauriae* and *A. brasilense* have some relatedness, but they are much more different on a whole genome level as could be deduced from phylogenetically used molecular marker genes. Whole genome sequencing is in progress to reveal more details—especially concerning traits present in the opportunistic pathogens as compared to the plant-associated strains.

116.5.4 Conclusion

In conclusion, results obtained by genome analysis investigations support findings gained through physiological and molecular studies discussed in the beginning of this chapter in terms of mechanisms shared by both plant-associated bacteria and those known as human pathogens. Genes encoding molecular systems and proteins with a potential ambivalent role are very often highly homologous, sometimes even nearly identical among these two groups of bacteria. Nevertheless, in some cases, specific physiological features from plant-associated bacteria give a hint about a possible threat these bacteria could pose in terms of causing diseases in humans. *S. rhizophila*, for instance, does certainly share several virulence-associated genes and gene clusters with human/animal pathogenic bacteria as described above, but it is incapable of growth at 37 °C, which is a crucial prerequisite for successful survival and virulence in the human body. The beneficial plant-associated *S. maltophilia* R551-3 strain, on the contrary, can grow at 37 °C, but it lacks genetic potentials similar to those discussed from *S. rhizophila*. However, naturally occurring gene communication mechanisms among bacteria such as horizontal gene transfer and recombination events could always equip the bacterial genome arsenal with new genetic information, leading to development of novel physiological traits. On the other hand, several studies have shown that plant- and human-associated bacteria harbor similar "interaction factors." Their mere occurrence in the genome is not necessarily an evidence for pathogenicity; there is a better correlation at protein level as shown for 14 epidemic bacterial killers ("badbugs") (Georgiades and Raoult, 2011). Therefore, beyond whole genomic and comparative bioinformatic analysis, proteomics and interaction studies seem to be necessary to assess the potential risk of opportunistic pathogenicity of bacterial strains.

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Queries in Chapter 116

Q1. Please check and confirm Schmid et al. 2010 has been changed to Schmid et al. 2009 so that this citation matches the list.

Q2. Ref Moulin et al. (2000) is given in the list but not cited in the text. Please cite it in the text or delete the same form the list.

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