Twitching Motility Is Essential for Endophytic Rice Colonization by the N₂-Fixing Endophyte *Azoarcus* sp. Strain BH72

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Azoarcus sp. strain BH72, as an endophyte of grasses, depends on successful host colonization. Type IV pili are essential for mediating the initial interaction with rice roots. In the genome sequence analysis, the *pilT* gene was identified, which encodes for a putative type IV pilus retraction protein. PilT of *Azoarcus* sp. BH72 shares high similarity to PilT of the human pathogen *Pseudomonas aeruginosa* PAO1 (77% amino acid sequence identity) and contains a predicted nucleotide-binding motif. To gain more insights into the role of the type IV pili in the colonization process of *Azoarcus* spp., we constructed an insertional mutant of *pilT* and a deletion mutant of *pilA*, the major structural component of the pilus structure. The *pilT* mutant, as the pilin deletion mutant Δ *pilA*, was abolished in twitching motility. Western blot analyses and electron microscopy studies demonstrated an enhanced piliation of the *Azoarcus pilT* mutant strain compared with the wild type, indicating that, indeed, PilT has a role in pilus retraction. Studies on rice root colonization in gnotobiotic cultures revealed that the establishment of microcolonies on the root surface was strongly reduced in the Δ *pilA* mutant, whereas the surface colonization was reduced by only 50% in the nontwitching *pilT* mutant. However, endophytic colonization of rice roots was strongly reduced in both mutants. These results demonstrate that the retractile force mediated by PilT is not essential for the bacterial colonization of the plant surface, but that twitching motility is necessary for invasion of and establishment inside the plant. Thus, a novel determinant for endophytic interactions with grasses was identified.

Azoarcus sp. strain BH72 is an endophytic diazotroph isolated from Kallar grass in Pakistan (Reinhold et al. 1986; Reinhold-Hurek et al. 1993b) that is capable of supplying its host with substantial amounts of fixed nitrogen (Hurek et al. 2002). It shows colonization and infection patterns of roots in gnotobiotic culture with its original host Kallar grass similar to those of rice (Hurek et al. 1994; Reinhold-Hurek and Hurek 1998a). Sites of primary colonization and entry into the plant are undifferentiated tissues above the root tips and the points of emergence of lateral roots (Reinhold-Hurek and Hurek 1998b). This endophyte is able to invade the central parts of the root (xylem) and to spread systemically into the shoot; however, the main colonization site is the root cortex, the aerenchyma (Hurek et al. 1994). Despite a dense colonization, no symptoms of plant disease and only very limited defense

responses are elicited in rice seedlings (Miché et al. 2006). *Azoarcus* sp. strain BH72 is capable of endophytic nitrogen fixation, expressing nitrogenase (*nifH*) genes inside the roots of field-grown Kallar grass (Hurek et al. 1997a) and rice grown in the laboratory (Egener et al. 1999; Reinhold-Hurek and Hurek 1998b). Only very little is known about mechanisms that mediate endophytic establishment of bacteria in grasses.

The ability to interact with the rice plant plays a critical role in the colonization by *Azoarcus* sp. strain BH72 (Dörr et al. 1998). A crucial step for a successful infection with a following colonization is the adhesion to the host. To perform this interaction, many bacteria use type IV pili. Type IV pili are filamentous cell appendages composed of small protein subunits (145 to 160 amino acids), termed pilin. A highly conserved N-terminal region and a short, positively charged leader peptide, which is cleaved off from prepilin, are characteristics of type IV pilins (Strom and Lory 1993). The N terminus of the pilin is composed of α -helices, which form the core of the pilus fiber. The outside of the pilus fiber is composed of β -sheets packed against the core (Forest and Tainer 1997) and an extended C-terminal tail (Mattick 2002). The multifunctional type IV pili are widespread in gram-negative bacteria ranging from pathogens, where they function as virulence factors, to environmental species such as the genera *Myxococcus*, *Synechocystis*, *Aquifex*, and *Shewanella* (Burrows 2005; Craig et al. 2004; Mattick 2002). Type IV pili mediate pathogen adherence to host cells, as in the case of *Pseudomonas aeruginosa*, *Neisseria meningitidis*, *N. gonorrhoeae*, and *Moraxella bovis* (Hahn 1997; Strom and Lory 1993). Additionally, they are involved in bacteriophage adsorption (Bradley 1974), modulation of target cell specificity (Jonsson et al. 1994), transformation competence (Fussenegger et al. 1997), DNA binding (van Schaik et al. 2005), social gliding (Wu and Kaiser 1995), and twitching motility (Bradley 1980; Darzins and Russell 1997).

Pili also play a role in beneficial plant–bacteria interactions. The endophyte *Azoarcus* sp. strain BH72 requires type IV pili, encoded by *pilAB*, to form microcolonies on rice roots and on fungal mycelia (Dörr et al. 1998). Interestingly, the *pilAB* locus encoding the genes essential for piliation is unusual in *Azoarcus* spp. The *pilA* gene is coding for a short prepilin with only 59 amino acids, which is unique for the β subclass of proteobacteria and is cotranscribed with *pilB*, whose gene product is an exported protein that also is essential for piliation (Dörr et al. 1998). The type IV pili of *Azoarcus* spp. and the recently reported endoglucanase (Reinhold-Hurek et al. 2006) are the only determinants for colonization of the endorhizosphere that have been described so far for diazotrophic grass endophytes.

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In addition to acting as attachment factors, type IV pili also mediate twitching motility, a flagellum-independent movement characterized by short, intermittent jerks (Bradley 1980). It can be detected on agar plates by monitoring colony expansion; the twitching zone appears as a thin halo surrounding the colony (Semmler et al. 1999). The retraction of the pili is the motive force for twitching motility (Bradley 1980) that is thought to be driven by filament disassembly mediated by PilT, a nucleotide-binding protein (Hobbs and Mattick 1993; Kaiser 2000; Mattick and Alm 1995). Mutation of the conserved *pilT* gene leads to a characteristic phenotype; bacteria are unable to move over surfaces but show hyperpiliation (Merz et al. 2000; Whitchurch and Mattick 1994; Whitchurch et al. 1991). Many bacteria use twitching motility to colonize surfaces (Mattick 2002). Among plant-associated bacteria, the pathogen *Xylella fastidiosa* is able to migrate via pilus-driven twitching motility downward in the host plant's vascular system against the direction of the transpiration stream (Meng et al. 2005).

Here, we analyze the role of twitching motility for the endophytic establishment of *Azoarcus* sp. strain BH72 in rice roots. We show that a hyperpiliated mutant in *pilT* (encoding the probable pilus retraction protein), which is defective for twitching motility, still is able to colonize the root surface but is unable to invade rice roots. Our findings suggest that twitching motility mediated by type IV pili is a key parameter for systemic colonization of rice plants by the endophyte *Azoarcus* sp. strain BH72.

RESULTS

Genome-based identification of a *pilT* gene.

During annotation of the *Azoarcus* sp. strain BH72 genome, a putative operon containing the two genes *pilT* and *pilU2* was identified (Krause et al. in press). PilT and PilU shared high similarity to the well-studied PilT and PilU proteins of the human pathogen *P. aeruginosa* PAO1 (PilT(NP_249086) PilU(AAG03785), 77 and 72% amino acid sequence identity, respectively). The *pilT* gene generally encodes the type IV pilus retraction protein (Mattick 2002), whereas the function of *pilU2* gene product is unknown. *pilT* of strain BH72 coded for a protein of 346 amino acids, that carried a domain of an AAA-ATPase superfamily (Pfam PF00437) (*E* value 1.76×10^{-3}) as other PilT proteins at amino acid residues 122 to 256.

Generation of *pilT* insertional mutant and *pilA* deletion mutant.

Previous studies demonstrated a role for type IV pili in the interaction of *Azoarcus* sp. BH72 with rice plants (Dörr et al. 1998). However, pili might act as part of the adhesion mechanism itself, or by mediating twitching motility for the colonization of the host plant. Therefore, we constructed an insertional mutant of the *pilT* gene (BH Δ *pilT*) (Fig. 1A) to analyze the function of the pilus retraction protein PilT, and an in-frame deletion mutant of *pilA* (BH Δ *pilA*) (Fig. 1B) to allow comparison with a pilus-defective mutant. The insertional mutant BH Δ *pilT* was generated by insertion of a kanamycin resistance cassette into the *Hind*III site of the truncated *pilT* gene of pSKpilT1/2 (amino acids 11 to 288 deleted). Electroporation of *Azoarcus* sp. BH72 cells with plasmid p Δ *pilT* yielded a chromosomal *pilT::Km^r* insertion by double allelic exchanges (Fig. 1A), which was confirmed by Southern blot analyses (data not shown). For the in-frame deletion mutant BH Δ *pilA* (amino acids 5 to 54 deleted), plasmid pJBLP13 (Dörr et al. 1998) was used. The upstream sequence of the truncated *pilA* gene was extended by insertion of the *Hinc*II fragment of pJBLP2 (Dörr et al. 1998), resulting in plasmid p Δ *pilAIII* (details discussed below). The insert was subcloned

into the pK18*mobsacB* suicide vector, resulting in p Δ *pilAIV*, to perform the *pilA* deletion (Fig. 1B) via sucrose selection (Kamoun et al. 1992). The BH Δ *pilA* mutant strain was confirmed by Southern blot (data not shown).

To exclude a polar effect of *pilT* mutagenesis on *pilU2* expression, semiquantitative mRNA analyses by reverse-transcriptase polymerase chain reaction (RT-PCR) were performed with RNA extracts of wild-type compared with BH Δ *pilT* cells. Samples of RT-PCR reactions were taken at different cycle numbers in order to avoid saturation of the PCR amplification (Egener et al. 2002) and were subjected to gel electrophoresis (Fig. 1C). As a control, 16S rRNA was used. To quantify the band intensities, three replicates and a statistical analysis were carried out. The *pilU2* fragments for cycle 34 were estimated and showed a 0.7- to 1.3-fold intensity for the mutant BH Δ *pilT*

Fig. 1. Schematic representation of the construction of **A**, the insertional mutant BH Δ *pilT* and **B**, the deletion mutant BH Δ *pilA*. Relevant restriction sites for endonucleases are: *Bam*HI (B), *Bbs*I (BI), *Bgl*II (BII), *Eco*RI (E), *Hinc*II (HII), *Hind*III (H), *Pst*I (P), and *Xho*I (X). **C**, Expression of *pilU2* and 16S rRNA genes of *Azoarcus* sp. strain BH72 (**A** and **C**) and BH Δ *pilT* (**B** and **D**), respectively. Reverse-transcriptase polymerase chain reaction analysis of total RNA extracted from late-exponential-phase cultures. At cycle numbers indicated below, aliquots of 5 μ l were taken and subjected to agarose gel electrophoresis. Corresponding controls without template and for DNA contamination of total RNA (heat-inactivated reverse transcriptase) taken after 34 cycles were negative (data not shown).

compared with the wild-type samples. The results demonstrated no significant difference in the expression level of the *pilU2* gene in the *pilT* mutant compared with the wild type (one sample *t* test, $P > 0.05$). The intensities of the BH Δ *pilT* 16S rRNA bands also did not differ significantly from the wild type (one sample *t* test, $P > 0.05$).

Twitching motility is abolished in *pilT* mutant.

In order to analyze the role of *pilT*, we tested the ability to perform twitching motility in the BH Δ *pilT* mutant strain compared with the wild type and the *pilA* deletion mutant BH Δ *pilA*. The wild type showed a flat "twitching zone" around the colonies, while BH Δ *pilT* and BH Δ *pilA* were defective in twitching motility (Fig. 2A): edges of the colonies were smooth and did not show the characteristic rugose and ruffled appearance of motile rafts of twitching (Darzins 1993) seen in wild-type colonies. Determination of colony areas in square units of 60 colonies per strain revealed a twofold reduction in the colony areas of mutants BH Δ *pilT* (448 ± 67) and BH Δ *pilA* (383 ± 83) compared with the wild type (980 ± 265) (Fig. 2B).

Enhanced piliation is observed in the *pilT* mutant.

To further characterize the phenotype of the *pilT* insertional mutant, we performed Western blot analyses and electron microscopy. Western blot analyses of whole-cell extracts with antisera against the structural subunits PilA and PilB indicated that *pilT* mutant and wild-type cells have a comparable level of cellular PilA and PilB proteins (Fig. 3A). In contrast, a crude pilus preparation of the extracellular fraction showed a strongly enhanced PilAB content in the mutant compared with the wild type (Fig. 3B). Thus, the *pilT* mutant appeared to be hyperpiliated. Interestingly, PilA was hardly detectable in the pilus preparation of wild-type cells in several experiments (not shown), indicating that the binding of the polyclonal antibody to the C-terminal peptide might be hindered by a posttranslational modification of PilA that is less pronounced in the *pilT* mutant.

Additionally, the piliation of *Azoarcus* sp. strain BH72 and BH Δ *pilT* were examined by transmission electron microscopy. Consistent with the results of the Western blot analyses, *pilT* mutant cells compared with the wild-type cells showed more pilus structures (Fig. 3D and C). Also, the crude preparation of extracellular pilus fibers confirmed this observation, because it

contained a much higher pilus density for the *pilT* mutant compared with the wild type (Fig. 3F and E).

PilT function is not obligate for bacterial colonization of the rice root surface.

It has been demonstrated previously that type IV pili are involved in the bacterial colonization of plant roots (Dörr et al. 1998). The points of emergence of lateral roots are a preferred site of root colonization and invasion by *Azoarcus* sp. strain BH72 (Hurek et al. 1994; Reinhold-Hurek and Hurek 1998b); therefore, the quantification of microcolonies at these sites is a good indicator for establishment of *Azoarcus* sp. on the root surface (Dörr et al. 1998). To test whether type IV pilus-mediated twitching motility is required for this colonization process

Fig. 2. Twitching motility assay of wild-type *Azoarcus* sp. BH72 and mutants. **A**, Light micrographs of single colonies grown under humid conditions for wild-type BH72, the pilin mutant BH Δ *pilA*, and the PilT-mutant BH Δ *pilT*. All panels are shown at the same level of magnification. **B**, Determination of the colony area in square units. Error bars above columns give standard deviations from 60 colonies per strain.

Fig. 3. Type IV pilus production by a *pilT* mutant. Immunoblot detection of PilA and PilB in **A**, whole-cell extract and **B**, crude preparations of extracellular pili obtained from equal amounts of cells or culture supernatants, respectively, from wild-type *Azoarcus* sp. strain BH72 (wt) or BH Δ *pilT* mutant. **C–F**, Piliation of *Azoarcus* wild-type and *pilT* mutant cells analyzed by transmission electron microscopy with negative staining. **C**, BH72 wild-type cells, **D**, *pilT* mutant cells; pilus preparation of culture supernatants from **E**, wild-type or **F**, *pilT* mutant cells. Black arrows indicate pili. Scale bars = 500 nm.

of rice roots, we inoculated rice seedlings with either wild-type BH72, the *pilA* mutant, or the *pilT* mutant strain under gnotobiotic conditions and determined the capacity of the bacteria to establish microcolonies on points of emergence of lateral roots within 2 weeks. Microcolonies of viable cells stained with a fluorescent dye (Dörr et al. 1998) were easily detectable for wild-type and *pilT* mutant cells, but rare in the *pilA* mutant strain, as expected. A statistical evaluation demonstrated that the *pilT* mutant colonized half as many points of emergence of lateral roots as the wild type, whereas colonization rates for the pilus mutant were very low (Fig. 4A). Taken together, this suggests that type IV pili are essential for the colonization of the plant surface, but twitching motility, the retractile force mediated by PilT, appears to be less important.

Twitching motility is required for endophytic colonization of rice roots.

To analyze whether the *pilT* mutant is not only able to attach to the plant surface but also to colonize the interior of the plant root, rice inoculation experiments similar to those above were carried out. For quantification of the invasion of bacteria into rice roots, bacteria attached to the surface were removed by ultrasonication (Reinhold-Hurek et al. 2006), and CFU released after homogenization of roots were counted per plant. Mutants of *pilA* and *pilT* showed a strongly reduced ability to invade the roots compared with the *Azoarcus* wild type (Fig. 4B). The number of endophytic wild-type cells differed significantly from cells of the *pilA* mutant ($P < 0.05$) and *pilT* mutant ($P < 0.01$). However, both mutants did not show a significant difference from each other ($P > 0.05$) (nonparametric analysis of variance by Kruskal-Wallis test). This indicates that twitching motility mediated by PilT is necessary for plant invasion by *Azoarcus* spp.

DISCUSSION

In this study, we elucidated the role of type IV pilus-mediated twitching motility in the colonization process of plants by the endophyte *Azoarcus* sp. strain BH72. Although the role of type IV pili is well documented in many human or animal pathogens, knowledge of plant-associated bacteria is more scarce. The relevance of type IV pili as a major determinant for successful colonization of rice roots by *Azoarcus* spp. was shown by mutational analyses of the *pilAB* operon that is essential for pilus formation (Dörr et al. 1998). However, in addition to pilus-mediated attachment, twitching motility is involved in colonization of host surfaces by pathogens such as *P. aeruginosa* or *N. gonorrhoeae* (Mattick 2002). In analogy, type IV pili also might function in movement on or in the plant tissue in a beneficial plant-microbe interaction and, thus, contribute to establishment and systemic spreading of the bacteria.

During annotation of the *Azoarcus* sp. BH72 genome, the *pilT* gene putatively coding for the pilus retraction protein was identified (Krause et al. 2006). PilT showed high sequence identity to the well-studied motor protein PilT of *P. aeruginosa*. Twitching motility is based on a mechanism which includes pilus extrusion, surface attachment of the pilus tip, and pilus retraction to convey the cell toward the point of adhesion (Merz and So 2000; Skerker and Berg 2001). ATPases are required to generate the mechanical force for pilus assembly and disassembly (Merz and Forest 2002; Wall and Kaiser 1999). The PilT ATPase is responsible for pilus retraction via disassembly of the pilus subunits at the base of the pilus fiber. Concordantly, the *Azoarcus* PilT harbors an ATPase domain. The PilB ATPase powers the extrusion process (Mattick 2002; Whitchurch et al. 1991). A paralogue of PilT, PilU is necessary for twitching motility but its function is still unclear (Burrows

2005; Whitchurch and Mattick 1994). Interestingly, in the genome of *Azoarcus* sp. strain BH72 three copies of *pilU* coding for an NTPase domain-containing protein were found (Krause et al. 2006). For *pilT* mutants of *Neisseria* spp. where PilQ, an outer membrane secretin responsible for the extrusion of the type IV pili through the outer membrane, was inactivated, a lethal phenotype was obtained due to unopposed pilus extension within the periplasm (Burrows 2005). In comparison, *pilQ pilU* mutants do not express ingrown pili (Wolfgang et al. 2000), indicating that PilU is not directly involved in pilus retraction. The role of three copies of *pilU* in *Azoarcus* spp. remains unclear.

To study the role of twitching motility in the interaction of a *Azoarcus* sp. with its host plant, we carried out insertional mutagenesis of the *pilT* gene. In an assay for twitching motility, strain BH72 exhibited typical flat twitching zones around the colonies, whereas the colonies of the *pilT* mutant and the pilin-deficient *pilA* mutant were smooth domed and showed no twitching zones. Therefore, the loss of the PilT ATPase resulted in a nontwitching mutant strain with apparently nonretractile pili.

Fig. 4. A, External and **B,** endophytic colonization of rice roots by wild-type *Azoarcus* sp. strain BH72 and mutant strains BHΔ*pilA* or BHΔ*pilT*. **A,** Establishment of microcolonies at the points of emergence of lateral roots. After growth of rice plants in gnotobiotic cultures for 13 days, roots were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Leiden, The Netherlands) and inspected for bacterial colonies by fluorescence microscopy. Percentage of colonized lateral roots was calculated and shown with standard deviations. For each strain, 15 to 23 emergence points of lateral roots out of six plants obtained in two independent experiments were inspected. Columns were significantly different from each other ($P < 0.05$) (nonparametric analysis of variance by Kruskal-Wallis test). **B,** Quantification of endophytic rice root colonization. Gnotobiotic rice cultures were grown as above for 12 days; then, roots were treated by ultrasonication and internal bacteria were quantified from homogenized roots by counting of CFU per plant (black bars with standard deviations). For each strain, rice roots of five plants were analyzed per experiment for two independent experiments. Columns of wild-type BH72 versus *pilA* mutant ($P < 0.05$) and *pilT* mutant ($P < 0.01$) were significantly different from each other. The difference between *pilA* and *pilT* mutants was not significant ($P > 0.05$) (nonparametric analysis of variance by Kruskal-Wallis test).

In *P. aeruginosa*, this phenotype is based on incapability to disassemble the pilus fiber subunits, which leads to a hyperpiliated variant (Whitchurch and Mattick 1994). Western blot analyses of the *Azoarcus* wild-type and *pilT* mutant with antibodies against the putative structural components of the type IV pilus, PilA and PilB, demonstrated an enhanced extracellular piliation, as expected. The finding that both PilA and PilB occurred in the pilus preparation further corroborates the assumption that both proteins are structural components of pili of *Azoarcus* sp. strain BH72. The prepilin PilA of strain BH72 is considerably shorter (59 amino acids) than most other pilins (approximately 150 to 170 amino acids), and the gene is co-transcribed with *pilB*, encoding a secreted protein that is essential for piliation (Dörr et al. 1998). However, such a pilus assembly is unusual because, typically, type IV pili are composed of only one structural protein, PilA. Interestingly, the PilA protein was hardly detectable in the pilus preparation of the wild-type cells. The anti-PilA polyclonal antibody had been raised against a C-terminal peptide of PilA (Dörr et al. 1998). It can be speculated that modifications of the extracellular pilin subunits might prevent antibody binding in the wild-type strain. Posttranslational modifications of pilin were described for *Pseudomonas* and *Neisseria* spp. which modify the surface characteristics and fiber morphology (Forest et al. 1999). A saturated modification pathway in the *pilT* mutant strain, resulting in the presence of unmodified and detectable PilA, may explain the difference in PilA detection. On the other hand, PilT might be directly required for modification of the pilin subunits, as was hypothesized for *Synechocystis* spp. (Bhaya et al. 2000).

The hyperpiliation of the *pilT* mutant of *Azoarcus* was confirmed by electron microscopy. Most probably, the mutation in the *pilT* gene resulted in a mutant with nonretractile pili, which lost the ability to disassemble the pilus subunits. This phenotype, characterized by hyperpiliation and a defect in twitching motility, also was described for *P. aeruginosa* (Bradley 1980; Whitchurch et al. 1991), *N. gonorrhoeae* (Merz et al. 2000), and *Myxococcus xanthus* (Sun et al. 2000). The phenotypical characterization of the *Azoarcus pilT* mutant suggests that the PilT function, the pilus retraction, not only is conserved in many gram-negative pathogens but also proceeds in nonpathogenic, plant-associated bacteria. Recent studies of *N. meningitidis* indicate a further role of PilT, being involved in the regulation of pilus-mediated adhesion (Yasukawa et al. 2006).

In order to address the questions of whether twitching motility is a part of the colonization process of *Azoarcus* spp., and in which phase the endophyte depends on the retractile force, rice inoculation studies were carried out. We observed a strong reduction in colonization of rice roots for a *pilA* deletion mutant, which confirmed previous results from an insertional *pilAB* mutant (Dörr et al. 1998). In contrast, the hyperpiliated, nonretractile *pilT* mutant of *Azoarcus* still was able to adhere to the plant surface, albeit with half the efficiency. However, it was strongly impeded in endophytic establishment in rice roots. This suggests that the attachment to the host depends on the binding of the type IV pili but not inevitably on the motile force. Twitching motility is required for the following step, the invasion of the rice root and establishment therein.

Compared with animal and human pathogens, fewer reports exist regarding plant-associated bacteria and the role of twitching motility in their interaction with the host plants. Only for the plant pathogens *Ralstonia solanacearum* and *Xylella fastidiosa* it is known that twitching motility is involved in colonization. The *pilT* mutant of *Ralstonia* spp. caused slower and less severe wilting on susceptible tomato plants (Liu et al. 2001). *Xylella* spp. use the type IV pili for twitching-mediated, downward migration in the host vascular system (Meng et al.

2005). Recent studies proved the enormous motile force of a single type IV pilus, which exceeds 100 pN in the human pathogen *N. gonorrhoeae* (Maier et al. 2002). Our results present the first observation of twitching motility and its role in a non-plant-pathogenic plant-associated bacterium. PilT-mediated twitching motility is essential for the endophytic lifestyle of *Azoarcus* sp. strain BH72 and, thus, one of the few known determinants for root infection by diazotrophic grass endophytes.

MATERIALS AND METHODS

Bacterial strains and plasmids.

The bacterial strains and plasmids used in this study are described in Table 1.

Media and growth conditions for bacteria.

Escherichia coli was grown in Luria broth (LB) or on LB agar plates (Ausubel et al. 1987) at 37°C. An *Azoarcus* sp. routinely was grown aerobically at 37°C in VM-ethanol medium or on VM-ethanol agar plates (Reinhold-Hurek et al. 1993a) and for electroporation and conjugation on VM-medium with malate instead of ethanol. Antibiotics used for *E. coli* or *Azoarcus* strains were tetracycline (12.5 µg ml⁻¹), kanamycin (30 µg ml⁻¹), and ampicillin (150 or 30 µg ml⁻¹). For plant inoculation, precultures were pregrown aerobically in VM-ethanol medium, then cells were washed and grown in SM medium supplemented with 0.01% yeast extract and 0.05% NH₄Cl (Reinhold et al. 1985). Cells with an optical density of 0.6 to 1.0 at 578 nm were washed twice in plant medium (Egener et al. 1999).

Plant inoculation studies.

Oryza sativa subsp. *indica* cv. IR36 was used. The rice grains were dehusked, surface sterilized, and germinated on agar plates. Plants were inoculated and grown as previously described (Egener et al. 1999).

For quantification of bacterial colonization inside the root, plants were harvested 12 days after inoculation. Roots were rinsed in distilled water and treated by ultrasonification for 15 min at room temperature in sterile distilled water (Reinhold-Hurek et al. 2006) in a Transonic T420 (Elma GmbH, Singen, Germany) with maximum output. After washing in sterile distilled water, they were homogenized by grinding, and diluted bacterial suspensions were grown in SM medium (Reinhold et al. 1985) agar plates supplemented with agar (8 g/liter) and yeast extract (20 mg/liter) for determination of CFU. For detection of lateral root colonization, plants were harvested 13 days after inoculation, rice roots were rinsed with distilled water and stained overnight in the dark at 4°C with the LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Leiden, The Netherlands), and emergence points of lateral roots were inspected for microcolonies by fluorescence microscopy.

DNA manipulations.

DNA manipulation, plasmid and genomic DNA extractions, transformation, Southern hybridization, and digoxigenin labeling of probe DNA were performed by using standard molecular biology techniques (Ausubel et al. 1987). The enzymes for DNA manipulation were supplied by Fermentas (St. Leon-Rot, Germany) and Roche (Grenzach-Wyhlen, Germany).

Mutagenesis.

The inactivation of the *pilT* gene was achieved by insertion of a kanamycin antibiotic resistance cassette into the coding region of the gene. Using PCR on primers pilT1for/rev and

*pilT2*for/rev (Table 1), upstream and downstream fragments were amplified which spanned only the first 33 bp and the last 176 bp of *pilT* for an in-frame deletion. The PCR products *pilT1* and *pilT2* were cloned into the pPCR-Script AmpSK(+) cloning vector (Stratagene, La Jolla, CA, U.S.A.), resulting in pPCR*pilT1/pilT2*. pPCR*pilT1* was digested with *Bam*HI and *Hind*III, pPCR*pilT2* with *Hind*III and *Xho*I, and both inserts were cloned into pBluescript II SK(+) (pSK*pilT1/2*). The kanamycin cassette from pUC4KIXX (Pharmacia, Uppsala, Sweden) was inserted into pSK*pilT1/2* via the *Hind*III restriction site. The orientation of the inserts was determined by restriction endonuclease digestion. The final construct p*ΔpilT* was transformed into *Azoarcus* sp. BH72 by electroporation, and the selection for double homologous recombinants took place via antibiotic resistance Km^r and Amp^r-. DNA of mutant strain BH*ΔpilT* was digested with *Bbs*I and *Hind*III for genotypic confirmation by Southern blot analysis, and the hybridization was carried out with a digoxigenin-labeled kanamycin probe.

In-frame deletion of *pilA* was carried out by using the sucrose selection system (Kamoun et al. 1992). To construct BH*ΔpilA*, the *Eco*RI-*Bgl*II fragment of pJBLP13 (Dörr et al. 1998) was subcloned into pUC19, resulting in p*ΔpilAI*. To achieve a more efficient homologous recombination event, the upstream region of the deleted *pilA* was prolonged via the *Hinc*II fragment of pJBLP2. The 2.35-kb insert of *ΔpilAII* was subcloned into pUC19 by *Hinc*II digest and the *Eco*RI-*Hind*III

fragment of *ΔpilAIII* was subcloned into vector pK18*mobsacB*. The constructs were examined by restriction digests and sequencing. The final p*ΔpilAIV* plasmid was conjugated into the *Azoarcus* genome by triparental mating with the helper strain *E. coli* (pRK2013) (Figurski and Helinski 1979). Transconjugants were selected on 6% sucrose media. Southern blot analysis was performed by digesting the genomic DNA with *Pst*I and hybridization with a digoxigenin-labeled *pilAB* probe to proof the correct integration into the *Azoarcus* genome.

DNA sequence analysis.

DNA sequencing was performed with the dideoxynucleotide chain termination method using the ALFexpress automated sequencer (GE Healthcare, Freiburg, Germany) by standard procedures (Hurek et al. 1997b). Sequence comparisons were analyzed using the Blast program (Altschul et al. 1990). Protein domains were predicted using Pfam (Bateman et al. 2004).

RT-PCR analysis of *pilU2* and 16S rRNA.

Total RNA was extracted from overnight cultures of *Azoarcus* with the pEQGold TriFast Extraction Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany). DNase I (Roche, Mannheim, Germany) treatment was followed by one chloroform-phenol-isoamylalcohol extraction before precipitation. RNA concentrations were estimated spectrophotometrically. Scanning of ethidium bromide-stained gels (Typhoon 8600

Table 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description or sequence ^a	Reference or source
Strains		
<i>Escherichia coli</i>		
DH5αF ⁺	F'recal endA1 hsdR17(rK ⁻ , mK ⁺) supE44 (λ-thi-1 relA1 ϕ80dlacZΔM15 Δ(lacZYA-argF) _{U169}	Hanahan, 1983
XL10-Gold Kan	Tet ^r Δ(<i>mcrA</i>)183 Δ(<i>mcrCB</i> - <i>hsdSMR</i> - <i>mrr</i>)173 <i>endA1 supE44 thi-1 recA1 gyrA96 relA1 lac</i>	Stratagene
ultracompetent cells	Hte [<i>proAB lacI</i> ^h ZΔM15 Tn10 (Tet ^r) Tn5 (Kan ^r) Amy]	
<i>Azoarcus</i> sp.		
BH72	Wild type	Reinhold et al. 1986
BHΔ <i>pilA</i>	Deletion of <i>pilA</i>	This study
BHΔ <i>pilT</i>	Km ^r , BH72 <i>pilT</i> ::Km cassette	This study
Plasmids		
pUC19	Ap ^r , ColE1 origin	Yanisch-Perron et al. 1985
pUC4KIXX	Ap ^r , Km ^r , source of Km cassette	Pharmacia
pPCR-Script AmpSK(+)	Ap ^r , ColE1 origin, fl1 (+) origin, <i>lacZ</i> ⁻ , P _{lac}	Stratagene
pBluescript II SK(+)	Ap ^r , ColE1 replicon	Stratagene
pRK2013	Km ^r , RK2 <i>tra</i> gene cloned in ColE1 replicon	Figurski and Helinski 1979
pK18 <i>mobsacB</i>	Km ^r , RP4 mob region, containing <i>sacB</i> gene	Schäfer et al. 1994
pJBLP13	Ap ^r , 4.6-kb <i>Eco</i> 47III- <i>Hind</i> III fragment of pJBLP1 in pJBP1, carrying <i>pilA</i> deletion	Dörr et al. 1998
pJBLP2	Ap ^r , <i>Eco</i> RI- <i>Sst</i> I fragment of pJBL2 in <i>Eco</i> RI- <i>Sst</i> I site of pUC19	Dörr and Reinhold-Hurek <i>unpublished</i>
pΔ <i>pilAI</i>	Ap ^r , 1.45-kb <i>Eco</i> RI- <i>Bgl</i> II fragment of pJBLP13 in pUC19 <i>Eco</i> RI- <i>Bam</i> HI	This study
pΔ <i>pilAII</i>	Ap ^r , 0.8-kb <i>Hinc</i> II fragment of pJBLP2 in <i>Hinc</i> II site of pΔ <i>pilAI</i>	This study
pΔ <i>pilAIII</i>	Ap ^r , 2.35-kb <i>Hinc</i> II fragment of pΔ <i>pilAII</i> in <i>Hinc</i> II site of pUC19	This study
pΔ <i>pilAIV</i>	Km ^r , 2.4-kb <i>Eco</i> RI- <i>Hind</i> III fragment of pΔ <i>pilAIII</i> in <i>Eco</i> RI- <i>Hind</i> III site of pK18 <i>mobsacB</i>	This study
pPCR <i>pilT1</i>	Ap ^r , 0.83-kb polymerase chain reaction (PCR) fragment containing first part of <i>pilT</i> gene in <i>Srf</i> I site of pPCR-Script	This study
pPCR <i>pilT2</i>	Ap ^r , 0.8-kb PCR fragment containing last part of <i>pilT</i> gene in <i>Srf</i> I site of pPCR-Script	This study
pSK <i>pilT1</i>	Ap ^r , 0.83-kb <i>Bam</i> HI- <i>Hind</i> III fragment of pPCR <i>pilT1</i> in pBluescript® II SK(+)	This study
pSK <i>pilT1/2</i>	Ap ^r , 0.8-kb <i>Hind</i> III- <i>Xho</i> I fragment of pPCR <i>pilT2</i> in pSK <i>pilT1</i>	This study
pΔ <i>pilT</i>	Ap ^r , Km ^r , 1.6-kb Km cassette in <i>Hind</i> III site of pSK <i>pilT1/2</i>	This study
Primers		
<i>pilT1</i> for	5'-GCGGATCCCACGAGCGTAGCCCCCTTC-3'	This study
<i>pilT1</i> rev	5'-CCAAGCTTCAGGTCGGAAGCCTTGTTCTTG-3'	This study
<i>pilT2</i> for	5'-GCAAGCTTGCCATCCGTAACCTGATCC-3'	This study
<i>pilT2</i> rev	5'-CCCTCGAGACACGATGCAGTTCTTGTGC-3'	This study
<i>pilU2</i> for	5'-AAGAACGGTTCGACCTCTT-3'	This study
<i>pilU2</i> rev	5'-AACGCGTCTTCGTAGCTGAT-3'	This study
1338r	5'-TTACTAGCGANTCCNNTTCANG-3'	Hurek et al. 1993
TH3	5'-GATTGGAGCGGCCGATGTC-3'	Hurek et al. 1993
TH5	5'-CTGGTTCGCCGAAGGCACCC-3'	Hurek et al. 1993

^a Ap^r, Km^r, and Tet^r = ampicillin, kanamycin, and tetracycline resistant, respectively.

Variable Mode Imager) and the corresponding software Image-Quant (GE Healthcare) were applied for accurate estimation of the band intensities. Forward and reverse primers (0.5 μ M) (Table 1) were applied for the RT-PCR reactions based on Ready-to-go RT-PCR beads (GE Healthcare). The RT step was done for 30 min at 42°C and 5 min at 95°C; cycling for 1 min at 95°C, 1 min (or 2 min for 16S rRNA) at the respective specific annealing temperature, and 1 min (or 2 min for 16S rRNA) at 72°C; followed by a 5-min final extension step at 72°C. The annealing temperature for the primers amplifying *pilU* and 16S rRNA were 58 and 70°C, respectively. The absence of DNA template was controlled by inactivation of the reverse transcriptase for 10 min at 95°C prior to the PCR reaction.

Twitching motility assay.

Cells of *Azoarcus* spp. from exponentially growing precultures were washed twice with 0.9% sodium chloride and diluted, and approximately 50 cells were plated on fresh VM-ethanol agar plates. Twitching plates were incubated under humid conditions by placing them into a plastic bag containing wet tissue papers. After incubation for 2 days at 37°C, they were transferred to room temperature for 2 to 4 days. Zones of twitching motility were examined via binocular microscope (Olympus SZX12). The colonies were scanned (Typhoon 8600 Variable Mode Imager), and the colony areas were measured by using the software Image Quant (GE Healthcare).

Pilus preparation.

Cells grown on VM-ethanol agar plates for 2 days at 37°C were scraped off, suspended in Tris-HCl buffer (50 mM, pH 8.0), and vigorously mixed to shear off cell appendages. Cells were removed by centrifugation (10,000 $\times$ g, 20 min) and the flagella were removed by ultracentrifugation (67,400 $\times$ g, 30 min) of the culture supernatant. An additional centrifugation step (100,000 $\times$ g, 3 h) yielded the pili pellet, which was suspended in Tris-HCl buffer (10 mM, pH 7.5) and stored at -20°C.

Western blot analysis.

For detection of the proteins, sodium dodecyl sulfate (SDS)-soluble cellular proteins from whole-cell extracts (Kiredjian et al. 1986) and pilus preparations were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (12.5% acrylamide) followed by immunoblotting (Hurek et al. 1994). Blots were incubated with rabbit polyclonal antibodies (1:5000) raised against PilA and PilB custom-synthesized peptides (amino acids 29 to 54 of PilA and amino acids 128 to 142 of PilB) (Eurogentec, Seraing, Belgium) (Dörr et al., 1998), and developed as described previously (Hurek et al. 1994) using a chemiluminescence reaction (ECL, Pierce, Erembodgem, Belgium).

Transmission electron microscopy.

Cells from twitching areas were deposited with a pipette tip in Tris-HCl buffer (10 mM, pH 7.5). The sample was transferred onto formvar- and carbon-coated copper grids (200 mesh) (Plano, Wetzlar, Germany). After 5 min of incubation, the liquid was removed with Whatman filters (Schleicher & Schuell, Dassel, Germany). Grids were stained for 5 min with 2% uranyl acetate and dried with Whatman filters. Pilus preparations were stained as above. The negatively stained grids were examined with a Zeiss EM 109 electron microscope. At least 10 fields of view were analyzed for each sample.

Statistical analysis.

The GraphPad InStat software package (GraphPad software, San Diego, CA) was used for statistical analysis.

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LITERATURE CITED

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403-410.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds. 1987. *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.
- Bateman, A., Coin, L., Durbin, R., Finn, R. D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S., Sonnhammer, E. L., Studholme, D. J., Yeats, C., and Eddy, S. R. 2004. The Pfam protein families database. *Nucleic Acids Res.* 32:D138-141. Database issue.
- Bhaya, D., Bianco, N. R., Bryant, D., and Grossman, A. 2000. Type IV pilus biogenesis and motility in the cyanobacterium *Synechocystis* sp. PCC6803. *Mol. Microbiol.* 37:941-951.
- Bradley, D. E. 1974. Basic characterization of a *Pseudomonas aeruginosa* pilus-dependent bacteriophage with a long non-contractile tail. *J. Virol.* 12:1139-1148.
- Bradley, D. E. 1980. A function of *Pseudomonas aeruginosa* PAO polar pili: Twitching motility. *Can. J. Microbiol.* 26:146-154.
- Burrows, L. L. 2005. Weapons of mass retraction. *Mol. Microbiol.* 57:878-888.
- Craig, L., Pique, M. E., and Tainer, J. A. 2004. Type IV pilus structure and bacterial pathogenicity. *Nat. Rev. Microbiol.* 2:363-378.
- Darzens, A. 1993. The *pilG* gene product, required for *Pseudomonas aeruginosa* pilus production and twitching motility, is homologous to the enteric, single-domain response regulator CheY. *J. Bacteriol.* 175:5934-5944.
- Darzens, A., and Russell, M. A. 1997. Molecular genetic analysis of type-4 pilus biogenesis and twitching motility using *Pseudomonas aeruginosa* as a model system—a review. *Gene* 192:109-115.
- Dörr, J., Hurek, T., and Reinhold-Hurek, B. 1998. Type IV pili are involved in plant-microbe and fungus-microbe interactions. *Mol. Microbiol.* 30:7-17.
- Egener, T., Hurek, T., and Reinhold-Hurek, B. 1999. Endophytic expression of *nif* genes of *Azoarcus* sp. strain BH72 in rice roots. *Mol. Plant-Microbe Interact* 12:813-819.
- Egener, T., Sarkar, A., Martin, D. E., and Reinhold-Hurek, B. 2002. Identification of a NifL-like protein in a diazotroph of the beta-subgroup of the *Proteobacteria*, *Azoarcus* sp. strain BH72. *Microbiology* 148:3203-3212.
- Figurski, D. H., and Helinski, D. R. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. *Proc. Natl. Acad. Sci. U.S.A.* 76:1648-1652.
- Forest, K. T., and Tainer, J. A. 1997. Type-4 pilus-structure: Outside to inside and top to bottom - a minireview. *Gene* 192:165-169.
- Forest, K. T., Dunham, S. A., Koomey, M., and Tainer, J. A. 1999. Crystallographic structure reveals phosphorylated pilin from *Neisseria*: Phosphoserine sites modify type IV pilus surface chemistry and fibre morphology. *Mol. Microbiol.* 31:743-752.
- Fussenegger, M., Rudel, T., Barten, R., Ryll, R., and Meyer, T. F. 1997. Transformation competence and type-4 pilus biogenesis in *Neisseria gonorrhoeae* - a review. *Gene* 192:125-134.
- Hahn, H. P. 1997. The type-4 pilus is the major virulence-associated adhesin of *Pseudomonas aeruginosa* - a review. *Gene* 192:99-108.
- Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166:557-580.
- Hobbs, M., and Mattick, J. S. 1993. Common components in the assembly of type 4 fimbriae, DNA transfer systems, filamentous phage and protein-secretion apparatus: A general system for the formation of surface-associated protein complexes. *Mol. Microbiol.* 10:233-243.
- Hurek, T., Burggraf, S., Woese, C. R., and Reinhold-Hurek, B. 1993. 16S rRNA-targeted polymerase chain reaction and oligonucleotide hybridization to screen for *Azoarcus* spp., grass-associated diazotrophs. *Appl. Environ. Microbiol.* 59:3816-3824.
- Hurek, T., Reinhold-Hurek, B., Van Montagu, M., and Kellenberger, E. 1994. Root colonization and systemic spreading of *Azoarcus* sp. strain BH72 in grasses. *J. Bacteriol.* 176:1913-1923.
- Hurek, T., Egener, T., and Reinhold-Hurek, B. 1997a. Divergence in nitrogenases of *Azoarcus* spp., *Proteobacteria* of the β -subclass. *J. Bacteriol.* 179:4172-4178.
- Hurek, T., Wagner, B., and Reinhold-Hurek, B. 1997b. Identification of N_2 -fixing plant- and fungus-associated *Azoarcus* species by PCR-based genomic fingerprints. *Appl. Environ. Microbiol.* 63:4331-4339.
- Hurek, T., Handley, L., Reinhold-Hurek, B., and Piché, Y. 2002. *Azoarcus*

- grass endophytes contribute fixed nitrogen to the plant in an unculturable state. *Mol. Plant-Microbe Interact.* 15:233-242.
- Jonsson, A.-B., Ilver, D., Falk, P., Pepose, J., and Normark, S. 1994. Sequence changes in the major pilus subunit of *Neisseria gonorrhoeae* leads to tropism variation to human tissue. *Mol. Microbiol.* 13:403-416.
- Kaiser, D. 2000. Bacterial motility: How do pili pull? *Curr. Biol.* 10:R777-R780.
- Kamoun, S., Tola, E., Kamdar, H., and Kado, C. I. 1992. Rapid generation of directed and unmarked deletions in *Xanthomonas*. *Mol. Microbiol.* 6:809-816.
- Kiredjian, M., Holmes, B., Kersters, K., Guilvout, I., and De Ley, J. 1986. *Alcaligenes piechaudii*, a new species from human clinical specimens and the environment. *Int. J. Syst. Bacteriol.* 36:286-287.
- Krause, A., Ramakumar, A., Bartels, D., Battistoni, F., Bekel, T., Boch, J., Böhm, M., Friedrich, F., Hurek, T., Krause, L., Linke, B., McHardy, A., Sarkar, A., Schneiker, S., Syed, A. A., Thauer, R., Vorhölder, F., Weidner, S., Pühler, A., Reinhold-Hurek, B., Kaiser, A., and Goesmann, A. 2006. Complete genome of the mutualistic, N₂-fixing grass endophyte *Azoarcus* sp. strain BH72. *Nat. Biotechnol.* 24:385-391.
- Liu, H., Kang, Y., Genin, S., Schell, M. A., and Denny, T. P. 2001. Twitching motility of *Ralstonia solanacearum* requires a type IV pilus system. *Microbiology* 147:3215-3229.
- Maier, B., Potter, L., So, M., Long, C. D., Seifert, H. S., and Sheetz, M. P. 2002. Single pilus motor forces exceed 100 pN. *Proc. Natl. Acad. Sci. U.S.A.* 99:16012-16017.
- Mattick, J. S. 2002. Type IV pili and twitching motility. *Annu. Rev. Microbiol.* 56:289-314.
- Mattick, J. S., and Alm, R. A. 1995. Common architecture of type 4 fimbriae and complexes involved in macromolecular traffic. *Trends Microbiol.* 3:411-413.
- Meng, Y., Li, Y., Galvani, C. D., Hao, G., Turner, J. N., Burr, T. J., and Hoch, H. C. 2005. Upstream migration of *Xylella fastidiosa* via pilus-driven twitching motility. *J. Bacteriol.* 187:5560-5567.
- Merz, A. J., and Forest, K. T. 2002. Bacterial surface motility: Slime trails, grappling hooks and nozzles. *Curr. Biol.* 12:297-303.
- Merz, A. J., and So, M. 2000. Interactions of pathogenic *Neisseriae* with epithelial cell membranes. *Annu. Rev. Cell. Dev. Biol.* 16:423-457.
- Merz, A. J., So, M., and Sheetz, M. P. 2000. Pilus retraction powers bacterial twitching motility. *Nature* 407:98-102.
- Miché, L., Battistoni, F., Gemmer, S., Belghazi, M., and Reinhold-Hurek, B. 2006. Differential colonisation and up-regulation of jasmonate-inducible defense proteins in roots of *Oryza sativa* cultivars upon interaction with the endophyte *Azoarcus* sp. *Mol. Plant-Microbe Interact* 19:502-511.
- Reinhold-Hurek, B., and Hurek, T. 1998a. Interactions of gramineous plants with *Azoarcus* spp. and other diazotrophs: Identification, localization and perspectives to study their function. *Crit. Rev. Plant Sci.* 17:29-54.
- Reinhold-Hurek, B., and Hurek, T. 1998b. Life in grasses: Diazotrophic endophytes. *Trends Microbiol.* 6:139-144.
- Reinhold, B., Hurek, T., and Fendrik, I. 1985. Strain-specific chemotaxis of *Azospirillum* spp. *J. Bacteriol.* 162:190-195.
- Reinhold, B., Hurek, T., Niemann, E.-G., and Fendrik, I. 1986. Close association of *Azospirillum* and diazotrophic rods with different root zones of Kallar grass. *Appl. Environ. Microbiol.* 52:520-526.
- Reinhold-Hurek, B., Hurek, T., Claeysens, M., and Van Montagu, M. 1993a. Cloning, expression in *Escherichia coli*, and characterization of cellulolytic enzymes of *Azoarcus* sp., a root-invading diazotroph. *J. Bacteriol.* 175:7056-7065.
- Reinhold-Hurek, B., Hurek, T., Gillis, M., Hoste, B., Vancanneyt, M., Kersters, K., and De Ley, J. 1993b. *Azoarcus* gen. nov., nitrogen-fixing proteobacteria associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth) and description of two species *Azoarcus indigenus* sp. nov. and *Azoarcus communis* sp. nov. *Int. J. Syst. Bacteriol.* 43:574-584.
- Reinhold-Hurek, B., Maes, T., Gemmer, S., Van Montagu, M., and Hurek, T. 2006. An endoglucanase is involved in infection of rice roots by the not cellulose-metabolizing endophyte *Azoarcus* sp. BH72. *Mol. Plant-Microbe Interact.* 19:181-188.
- Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., and Pühler, A. 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: Selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 145:69-73.
- Semmler, A. B., Whitchurch, C. B., and Mattick, J. S. 1999. A re-examination of twitching motility in *Pseudomonas aeruginosa*. *Microbiology* 145 (Pt 10):2863-2873.
- Skerker, J. M., and Berg, H. C. 2001. Direct observation of extension and retraction of type IV pili. *Proc. Natl. Acad. Sci. U.S.A.* 98:6901-6904.
- Strom, M. S., and Lory, S. 1993. Structure-function and biogenesis of the type IV pili. *Annu. Rev. Microbiol.* 47:565-596.
- Sun, H., Zusman, D. R., and Shi, W. 2000. Type IV pilus of *Myxococcus xanthus* is a motility apparatus controlled by the *frz* chemosensory system. *Curr. Biol.* 10:1143-1146.
- van Schaik, E. J., Giltner, C. L., Audette, G. F., Keizer, D. W., Bautista, D. L., Slupsky, C. M., Sykes, B. D., and Irvin, R. T. 2005. DNA binding: A novel function of *Pseudomonas aeruginosa* type IV pili. *J. Bacteriol.* 187:1455-1464.
- Wall, D., and Kaiser, D. 1999. Type IV pili and cell motility. *Mol. Microbiol.* 32:1-10.
- Whitchurch, C. B., and Mattick, J. S. 1994. Characterization of a gene, *pilU*, required for twitching motility but not phage sensitivity in *Pseudomonas aeruginosa*. *Mol. Microbiol.* 13:1079-1091.
- Whitchurch, C. B., Hobbs, M., Livingston, S. P., Krishnapillai, V., and Mattick, J. S. 1991. Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. *Gene* 101:33-44.
- Wolfgang, M., van Putten, J. P., Hayes, S. F., Dorward, D., and Koomey, M. 2000. Components and dynamics of fiber formation define a ubiquitous biogenesis pathway for bacterial pili. *EMBO (Eur. Mol. Biol. Organ.) J.* 19:6408-6418.
- Wu, S. S., and Kaiser, D. 1995. Genetic and functional evidence that type IV pili are required for social gliding motility in *Myxococcus xanthus*. *Mol. Microbiol.* 18:547-558.
- Yanisch-Perron, C., Vieira, J., and Messing, J. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33:103-119.
- Yasukawa, K., Martin, P., Tinsley, C. R., and Nassif, X. 2006. Pilus-mediated adhesion of *Neisseria meningitidis* is negatively controlled by the pilus-retraction machinery. *Mol. Microbiol.* 59:579-589.