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Type IV pili are involved in plant–microbe and fungus–microbe interactions

Juliane Dörr, Thomas Hurek and Barbara Reinhold-Hurek*

Max-Planck-Institut für terrestrische Mikrobiologie, Arbeitsgruppe Symbioseforschung, Karl-von-Frisch-Str., D-35043 Marburg, Germany.

Summary

Adherence of bacteria to eukaryotic cells is essential for the initiation of infection in many animal and human pathogens, e.g. *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa*. Adhesion-mediating type IV pili, filamentous surface appendages formed by pilin subunits, are crucial virulence factors. Here, we report that type IV pilus-dependent adhesion is also involved in plant–bacteria and fungus–bacteria interactions. Nitrogen-fixing, endophytic bacteria, *Azoarcus* sp., can infect the roots of rice and spread systemically into the shoot without causing symptoms of plant disease. Formation of pili on solid media was dependent on the *pilAB* locus. *PilA* encodes an unusually short (6.4 kDa) putative pilin precursor showing 100% homology to the conserved N-terminus of the *Pseudomonas aeruginosa* type IV pilin. *PilB* encodes for a 14.2 kDa polypeptide showing similarity to FimF, a component of type I fimbriae of *Escherichia coli*. It was found to be extruded beyond the cell surface by immunofluorescence studies, and it may, therefore, be part of a pilus assembly complex or the pilus itself. Both genes are involved in the establishment of bacteria on the root surface of rice seedlings, as detected by fluorescence microscopy. Moreover, both genes are necessary for bacterial adhesion to the mycelium of an ascomycete, which was isolated from the same rhizosphere as the bacteria. In co-culture with the fungus, *Azoarcus* sp. forms complex intracytoplasmic membranes, diazosomes, which are related to efficient nitrogen fixation. Adhesion to the mycelium appears to be crucial for this process, as diazosomes were absent and nitrogen fixation rates were decreased in *pilAB* mutants in co-culture.

Received 3 December, 1997; revised 4 June, 1998; accepted 8 June, 1998. *For correspondence. E-mail reinhold@mailer.uni-marburg.de; Tel. (6421) 178101; Fax (6421) 178109.

Introduction

The first crucial event in the infectious process of many animal and human pathogenic bacteria is the attachment to epithelial cells of the host. Type IV pili play an essential role in mediating bacterial adherence to and colonization of host cell surfaces and are virulence factors in *Neisseria gonorrhoeae*, *N. meningitidis*, *Pseudomonas aeruginosa* and *Moraxella bovis* (Strom and Lory, 1993; Hahn, 1997). In addition, type IV pili are involved in the modulation of target cell specificity (Jonsson *et al*., 1994), bacteriophage adsorption (Bradley, 1974), twitching motility (Bradley, 1980; Darzins and Russell, 1997), transformation competence (Fusenegger *et al*., 1997) and social gliding in bacteria (Wu and Kaiser, 1995).

Type IV pili are filamentous cell appendages of 6 nm thickness assembled primarily from a single protein subunit, pilin, which consists of approximately 150 amino acid residues. A short, positively charged leader peptide, which is cleaved off the prepilin, and a highly conserved N-terminal region are characteristic of type IV pilins (Strom and Lory, 1993). The hydrophobic N-terminal region is postulated to form the centre of the pilus by hydrophobic interactions of 54-amino-acid α-helices of the pilin subunits, whereas the C-terminal part is exposed to the pilus surface (Parge *et al*., 1995; Forest and Tainer, 1997). This hypervariable region is the most highly exposed portion (Forest and Tainer, 1997) and the source of strain-specific antigenic variation and interaction with host surfaces (Nassif *et al*., 1993). Additional proteins located at the tip of the filament, adhesins, may also interact with host receptors in *Neisseria* (Rudel *et al*., 1995).

To date, the involvement of type IV pili in adhesion has been described for microbe–microbe (Wu and Kaiser, 1995) or animal/human–microbe interactions only. Here, we report for the first time evidence for a more general role of type IV pili, also mediating attachment of bacteria to plant roots and fungal mycelium.

Nitrogen-fixing, Gram-negative bacteria of the genus *Azoarcus* occur in association with grasses (Reinhold-Hurek *et al*., 1993). They show similar colonization and infection patterns to their original host Kallar grass and rice seedlings in the laboratory (Hurek *et al*., 1994b; Reinhold-Hurek and Hurek, 1998a). Undifferentiated tissues above the root tips and the points of emergence of lateral roots are the sites of primary colonization of and entry into the plant (Reinhold-Hurek and Hurek, 1998b). Without

causing symptoms of plant disease, these endophytes are capable of invading the central parts of the root (xylem) and of systemic spreading into the stele. *In situ* hybridization studies have recently demonstrated that *Azoarcus* nitrogenase genes can be expressed inside the roots of field-grown Kallar grass (Hurek *et al*., 1997a). *Azoarcus* also interacts with rhizosphere fungi (Hurek *et al*., 1995; 1997b). In co-culture with an asexual ascomycete originating from Kallar grass roots, *Azoarcus* sp. strain BH72 adheres tightly to the actively respiring mycelium. Under these conditions, the bacteria form novel intracytoplasmic membrane stacks, diazosomes (Hurek *et al*., 1995), that are related to a high efficiency of nitrogen fixation (Hurek *et al*., 1994b). Here, we present evidence that type IV pili are involved in the colonization of both plant and fungal host surfaces, indicating that there may be common traits in the initial steps of interactions of bacteria with eukaryotic organisms of their habitat.

Results

A short type IV pilin is encoded in the pilAB *locus of* Azoarcus *sp. BH72*

Mutant BH1599, which had been obtained by transposon mutagenesis from wild-type *Azoarcus* sp. BH72, showed a pleiotropic phenotype: it was affected in attachment to plant roots and fungal mycelium (see below). Sequence analysis of the site of transposon insertion revealed two open reading frames (ORFs), *pilA* and *pilB*, the transposon disrupting *pilA* (Fig. 1A and B). Upstream of *pilA*, sequences homologous to σ^{54}-dependent promoters and potential binding sites for the transcriptional activators NifA and NtrC were located (Fig. 1B). Northern blot analysis with a *pilAB* gene probe indicated that both genes were co-transcribed as a 1.1 kb transcript (Fig. 1C). The N-terminus of the deduced protein PilA showed high homology with type IV prepilins, being 100% identical to a *Pseudomonas aeruginosa* prepilin in amino acids 5–30 (Fig. 2A). The *pilA* ORF was unusually short (59 amino acids, 6.4 kDa) in

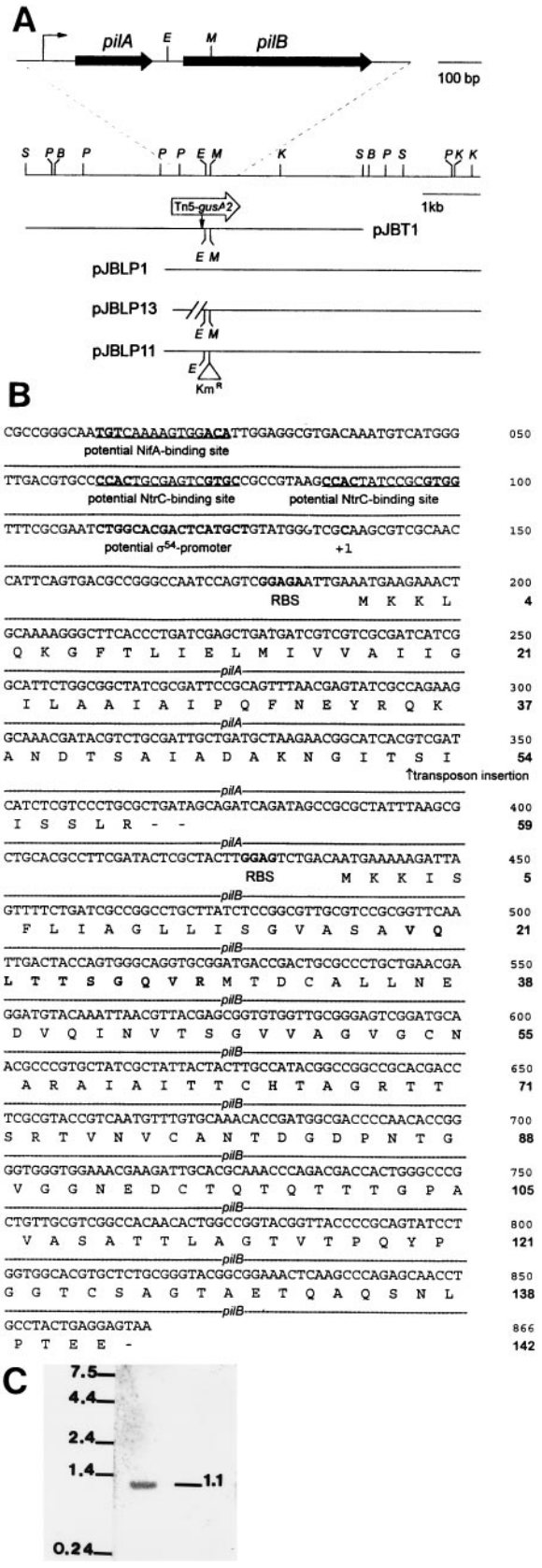

Fig. 1. Gene organization of the *pilAB* locus.
A. Restriction map of the *pilAB* locus. Small arrow indicates the putative promoter region. Inserts of plasmids used for localization of the transposon insertion (pJBT1) and complementation are given as lines. Relevant restriction sites are S, *Sph*I; P, *Pst*I; B, *Bam*HI; E, *Eco*47III; M, *Mfe*I; K, *Kpn*I. Tn*5*-gusA2, insertion site of transposon; Km^R, insertion site of Km-resistance cartridge. Tn*5* and Km^R appear at a smaller scale.
B. Sequence of *pilA* and *pilB* and the putative promoter region. Potential NifA, NtrC, σ^{54} and ribosome binding sites (RBS), the putative transcriptional start (+1), numbering for deduced amino acids, and amino acids determined by N-terminal amino acid sequencing of PilB are printed in bold.
C. Northern blot hybridized with a digoxigenin-labelled *pilAB* gene probe; sizes in kb.

comparison with prepilins of *P. aeruginosa* (149 amino acids) or *N. gonorrhoeae* (166 amino acids) (Fig. 2A). This result was confirmed by the sequencing of several different clones retrieved from a genomic library and of clones from a cDNA library constructed from mRNA of *Azoarcus* sp. BH72. Sequencing errors could also be excluded, as multiple stop codons were present in two reading frames. In the reading frame of *pilA*, stop codons were located at nucleotide positions 367, 370, 382, 433, 439, 502, 526 and 544. In frame +1, they were located at nucleotide positions 395, 449 and 563. In frame −1, another gene was identified (*pilB*).

In order to screen for additional gene copies of *pilA* that might have the normal size of a pilin gene, we carried out Southern hybridization studies of genomic DNA with an oligonucleotide probe corresponding to the conserved region (nucleotides 207–233) of *pilA*. Only one hybridizing fragment could be detected at high stringency (72°C) (Fig. 3) and, thus, no other identical gene copy was present in the genome of *Azoarcus* sp. BH72. Hybridization at lower stringency (60°C) yielded an additional hybridizing fragment (1.0 kb in *Pst*I-digested DNA, lane 4 in Fig. 3), which might represent a gene with a homologous but not identical 5′ end. However, cloning and sequencing of this fragment showed that neither a pilin nor a pseudopilin gene was encoded by the oligonucleotide-binding region (not shown; J. Plessl and B. Reinhold-Hurek, unpublished results).

An additional small protein PilB (14.2 kDa) was encoded downstream of *pilA*. The presence of a second protein that was not fused to PilA was confirmed by two-dimensional gel electrophoresis. A protein that was missing in the protein extract of mutant BH1599 in comparison with the wild type (Fig. 4) was identified by its N-terminal amino acid sequence. The analysed sequence, VQLTTSGQVR, was identical to amino acids 20–29 of the deduced sequence of PilB (Fig. 1B). This would be expected to be the N-terminus of mature PilB after cleavage of a signal peptide typical of secreted proteins. This was in accordance with the observation that the first 19 amino acids of the deduced PilB sequence fulfilled the criteria proposed by von Heijne (1988) for secretory signal peptides.

PilB showed some similarity to FimF of *E. coli* (Fig. 2B), which is a minor component of type I fimbriae. For cellular localization of PilB, polyclonal antibodies were raised against a C-terminal peptide of PilB, the preimmmune serum showing no cross-reaction (not shown). The antibodies specifically detected a protein with a slightly different electrophoretic mobility (21 kDa) than the calculated

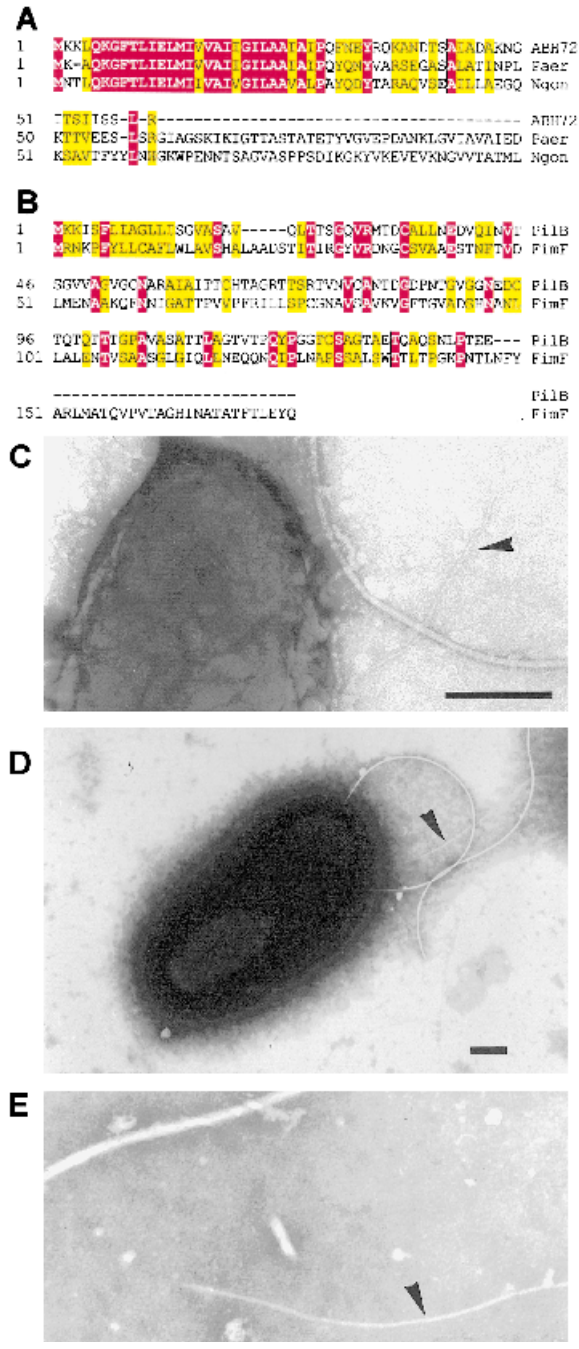

Fig. 2. Pilin genes and pili in *Azoarcus* sp. BH72.
A. Multiple alignment of the deduced amino acid sequence of *pilA* of *Azoarcus* sp. BH72 (ABH72, GenBank acc. no. AF031954) and prepilins of *Pseudomonas aeruginosa* (Paer, acc. no. PIR A25023) and *Neisseria gonorrhoeae* (Ngon, acc. no. PIR YQNHG). Amino acid residues identical or similar in all three proteins are boxed in purple or yellow respectively. Bulky hydrophobic (FILMVWY), aliphatic hydrophobic (ACILMV), positively charged (HKR), negatively charged and their derivatives (DENQ), small (ACGPST) amino acids were considered to be similar.
B. Alignment of *Azoarcus* PilB and *E. coli* FimF (acc. no. PIR S56543). Colours as in (A).
C–E. Transmission electron micrographs showing pili and flagella of a negatively stained *Azoarcus* sp. BH72 cell (C), a complemented mutant cell (BH1599*pilAB*) (D) or pilus and flagellum fragments of a *pilB* complementation mutant (BH1599*pilB*) (E). Pili are marked with an arrowhead. Bar = 0.2 μm.

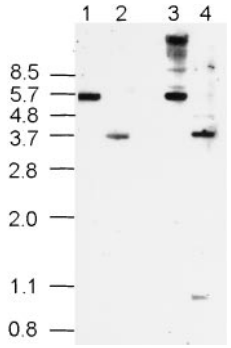

Fig. 3. Genomic fragments homologous to type IV pilin genes. Southern hybridization of genomic DNA of *Azoarcus* sp. BH72 digested with *Bam*HI (lanes 1 and 3) or *Pst*I (lanes 2 and 4) and hybridized with an oligonucleotide directed to the conserved 5′ end of *pilA* at high stringency (72°C; lanes 1 and 2) or low stringency (60°C; lanes 3 and 4). Size marker given in kb.

molecular mass, which might result from tertiary structure. PilB was found in cell extracts of *E. coli* expressing *pilAB* and of wild-type *Azoarcus* sp. BH72 (Fig. 5A and B), but not in extracts of mutant BH1599 (Fig. 5A and B). When *pilA* or *pilB* were complemented in the transposon mutant (see below), PilB was not detected in extracts of BH1599*pilA* (Fig. 5A). In a complementation mutant harbouring intact *pilB* but an in frame deletion of *pilA*, the PilB protein was still present in protein extracts and of the same size as in wild-type cells (Fig. 5A, lanes 3 and 2). If a hypothetical PilAB fusion protein existed, the in frame deletion would result in a reduction of protein size of 5.5 kDa, which should be clearly visible at an electrophoretic mobility of 20 kDa. Besides the sequencing data, this confirms that *pilA* and *pilB* are distinct genes encoding different proteins in *Azoarcus* sp. BH72.

A higher molecular mass form of PilB (60 kDa), possibly indicating protein complexes, was present in a crude fraction of proteins of cell appendages of wild-type *Azoarcus* (Fig. 5B), but not in extracts heated with reducing agents (Fig. 5B) or in external fractions of mutant BH1599 (not shown). In the cell appendage fraction of wild-type cells, PilB appeared to be enriched in comparison with the fraction of total cellular proteins (Fig. 5B). Immunofluorescence

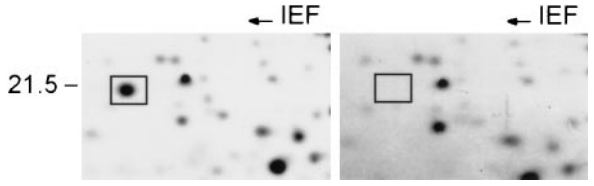

Fig. 4. Identification of PilB by two-dimensional gel electrophoresis. Extracts of total cell proteins of *Azoarcus* sp. BH72 (left) and mutant BH1599 (right). Close-up of isoelectric focusing (IEF) in the pH range 3.5–10 followed by an SDS–PAGE (12.5% acrylamide); marker given in kDa. Protein spot missing in strain BH1599 is boxed.

studies on whole cells showed specific binding of PilB antiserum to microcolonies of strain BH72 (Fig. 5C–E). PilB appeared to be extruded beyond the cell surface. Typically, not the entire cells were stained, but localized regions adjacent to bacterial cells or between bacterial cells (Fig. 5F).

Similar experiments were not carried out with antiserum to PilA, because an attempt to raise polyclonal antibodies against a C-terminal peptide of PilA did not result in specific cross-reactions with cell extracts.

Pilus formation on solid media is dependent on pilA *and* pilB

Wild-type *Azoarcus* cells cultivated on agar surfaces were inspected for the presence of pili by transmission electron microscopy. In addition to bacterial flagella, thinner filaments of the typical diameter of pili (approximately 4.5–6 nm) were visible (Fig. 2C). However, strain BH72 was

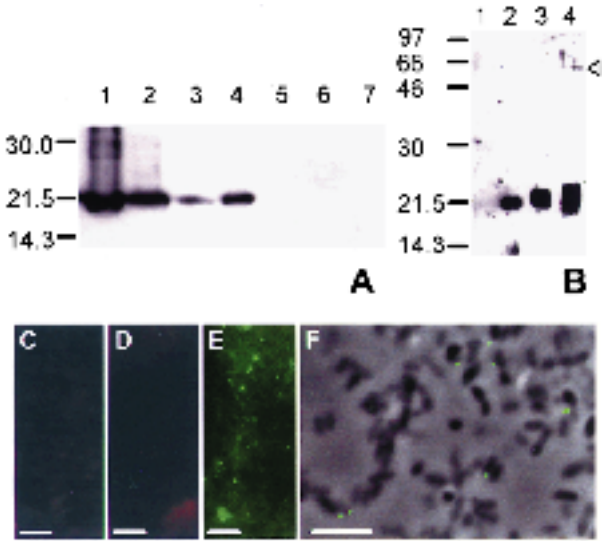

Fig. 5. Western blot and immunofluorescence analysis of PilB.
A and B. Western blot analysis of specificity of PilB antibodies. SDS extracts of cells of *E. coli* carrying *pilAB* on pJPP1 (lane A1) or cloning vector pUC19 (lane A7), of *Azoarcus* sp. BH72 (lanes A2 and B2), and of *Azoarcus* mutants BH1599 (A6 and B1), BH1599*pilB* (A3), BH1599*pilAB* (A4) and BH1599*pilA* (A5). Crude fraction of proteins of cell appendages of strain BH72 was heated (95°C) for 20 min (B3) or 2 min (B4) in the presence of SDS. An arrowhead marks a putative protein complex containing PilB. Loaded protein was 1 μg. Blots were incubated with PilB antiserum (1:5000) and developed by chemiluminescence (ECL, Amersham) to enhance sensitivity. Size markers given in kDa.
C–F. Localization of PilB by immunofluorescence on whole cells of *Azoarcus* sp. BH72 (C, E and F) or mutant BH1599 (D). Bacterial colonies from agar plates were attached to slides and incubated with preimmune serum (C) or PilB antiserum (D–F).
C–E. Original fluorescence microscopic images not processed for haze reduction and taken with identical RGB setting and exposure time.
F. Light microscopic phase-contrast image overlaid with fluorescence microscopic image. Bars = 5 μm.

Table 1. Bacterial strains, cloning vectors and recombinant plasmids used.

Strain, vector, plasmid	Relevant genotype or properties	Source or reference
Strains		
E. coli		
DH5α′	F′ *recA1 endA1 hsdR17*(r_K^-, m_K^+) *supE44* (λ^- *thi*-1 *relA1* ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)$_{U169}$	Hanahan (1983)
MC1061	*hsdR araD139* Δ(*araABC-leu*)$_{7679}$ Δ(*lac*)$_{X74}$ *galU* galK rpsL thi	Meissner *et al*. (1987)
Azoarcus sp.		
BH72	Wild type	Reinhold *et al*. (1986)
BH1599	Km^R, Tc^R, BH72 *pilA*::Tn*5-gusA2*	B. Reinhold-Hurek *et al*. unpublished
BH1599*pilAB*	Km^R, Tc^R, Ap^R, BH1599::pJBLP1, BH1599 complemented with *pilA* and pilB	This study
BH1599*pilA*	Km^R, Tc^R, Ap^R, BH1599::pJBLP11, BH1599 complemented with *pilA*	This study
BH1599*pilB*	Km^R, Tc^R, Ap^R, BH1599::pJBLP13, BH1599 complemented with *pilB*	This study
Cloning vectors		
pUC19	Ap^R, ColE1 origin	Yanisch-Perron *et al*. (1985)
pBK-CMV	Km^R, Neo^R, ColE1 origin, f1(-) origin, SV40 origin	Stratagene
Recombinant plasmids		
pJBL1	Km^R, Neo^R, *pilAB* on a 5.4 kb chromosomal *Bam*HI fragment of strain BH72, in pBK-CMV	This study
pJBLP1	Ap^R, insert of pJBL1 cloned in pUC19	This study
pJBLP11	Ap^R, Km^R, Km^R insertion in *pilB* of pJBLP1	This study
pJBP1	Ap^R, 0.35 kb PCR fragment containing a 153 bp deletion in *pilA*	This study
pJBLP13	Ap^R, 4.6 kb *Eco*47III/*Hin*dIII[a] fragment of pJBLP1 cloned in *Eco*47III/*Hin*dIII restriction site of pJBP1	This study
pJBT1	Ap^R, Km^R, Tc^R, *pilA*::Tn*5-gusA2* on a 14.7 kb chromosomal fragment of strain BH1599, in pUC19	This study
pJBT111	Ap^R, 1.6 kb *Sma*I[b]/*Sma*I fragment from pJBT1 in pUC19	This study
pJBT121	Ap^R, 1.5 kb *Kpn*I/*Hin*cII[b] fragment from pJBT1 in pUC19	This study
pJBT1112	Ap^R, 0.43 kb *Pst*I fragment from pJBT111 in pUC19	This study
pJPP1	Ap^R, 0.95 kb PCR fragment containing *pilAB* under the control of the *lac* promoter in pUC19	This study

a. Restriction site in polylinker of cloning vector.
b. Restriction site of Tn*5-gusA2*.

only sparsely piliated when grown on solid medium, mostly showing only one pilus per cell. Moreover, the pili were often broken and thus appeared to be very fragile. Pili were not detectable in colonies of mutant BH1599 when more than 200 cells were inspected. To investigate whether both genes (*pilAB*) are involved in pilus formation, complementation mutants of strain BH1599 were constructed by stable integration of plasmids into the chromosome via homologous recombination. Either the intact operon under the control of its own promoter was integrated (strain BH1599*pilAB*) or the integrated plasmid carried intact *pilA* and a disrupted *pilB* (strain BH1599*pilA*) or an in frame deletion of *pilA* and intact *pilB* (strain BH1599*pilB*) (Fig. 1A, Table 1). Electron microscopic inspection revealed that pilus formation was restored by complementation with both genes, *pilAB*. The wild-type piliation level (in 16% ± 10% of cells piliation visible) was achieved in mutant BH1599*pilAB* (20% ± 6%) (Fig. 2D). No pili were detected after complementation with *pilA* alone when 150 cells of BH1599*pilA* were inspected. Similarly, no cell-bound pili were detected after complementation with *pilB* (150 cells of BH1599*pilB*); however, fragments of pili were rarely detected on the grid (Fig. 2E), demonstrating that pili were formed, albeit at a very low rate. Pilus fragments not associated with cells were not detected on grids of mutants BH1599 or BH1599*pilA*. Apparently, PilA being truncated by 7 terminal amino acids in mutant BH1599 was sufficient for an (inefficient) pilus formation, when PilB was present. This also confirms the contribution of two distinct genes, *pilA* and *pilB*, to pilus formation, as they were transcribed from two physically separated fragments on the chromosome in this experiment.

A type IV pilin is involved in bacterial adhesion to the fungal mycelium and nitrogen fixation in co-culture with the rhizospheric asexual ascomycete

In co-culture with the rhizosphere fungus 2003 related to the *Euascomycetes*, most cells of *Azoarcus* sp. strain BH72 adhere to the fungal mycelium, where they form intracytoplasmic membrane stacks related to efficient nitrogen fixation (Hurek *et al*., 1995). During the first 2 days of incubation, the turbidity of the culture medium decreases because of adhesion of the bacterial inoculum to the mycelium, where bacterial microcolonies can be detected by using a fluorescent vitality stain. As fungal spores obscure

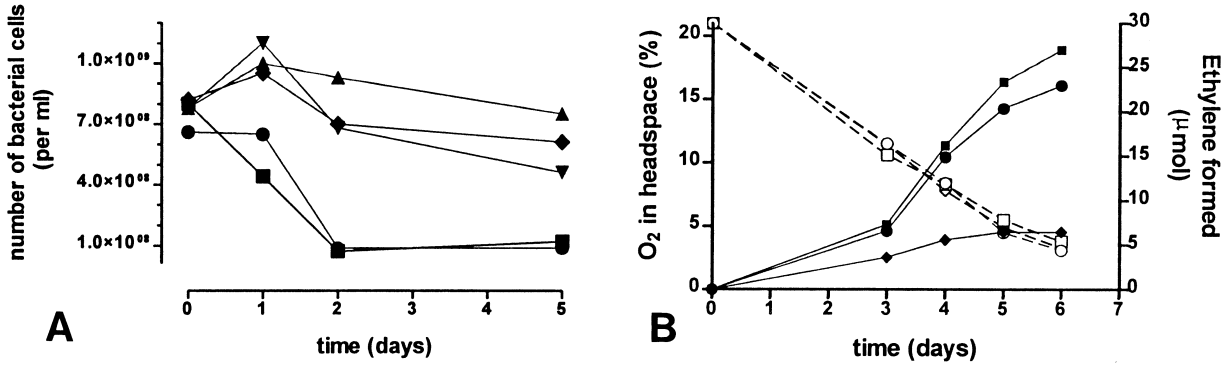

Fig. 6. Nitrogen fixation and attachment to fungal mycelium of *Azoarcus* sp. wild type and pilin mutants in co-culture with the asexual ascomycete 2003.
A. Time course of decrease in bacterial number in supernatant caused by attachment.
B. Time course of nitrogen fixation measured as ethylene accumulation by acetylene reduction in cultures having parallel profiles of oxygen consumption. (●), Wild-type BH72; (♦), transposon mutant BH1599; (■), complementation mutant BH1599*pilAB*; (▲), complementation mutant BH1599*pilB*; (▼), complementation mutant BH1599*pilA*.

turbidity measurements, bacterial numbers in the supernatant were determined by direct microscopic counts. Typical examples for the decrease in bacterial numbers are shown in Fig. 6A. The initial attachment within 2 days was significantly inhibited by disruption of either *pilA* or *pilB* or both, whereas the attachment of the transposon mutant was rescued by *pilAB*. Cell numbers present in the supernatant (in percentage of the initial inoculum as 100%) were $13.0 \pm 5.4\%$ (a) for wild-type strain BH72, $82.9 \pm 8.9\%$ (b) for transposon mutant BH1599, $10.1 \pm 2.1\%$ (a) for complementation mutant BH1599*pilAB*, $77.4 \pm 4.9\%$ (b) for complementation mutant BH1599*pilA* and $78.5 \pm 24.2\%$ (b) for complementation mutant BH1599*pilB*. Results are means ± standard deviations for four different cultures and are followed by different letters when they differ from each other significantly ($P < 0.001$).

The inactivation of the *pilAB* genes also affected nitrogen fixation and differentiation in association with the fungal mycelium. Figure 6B shows typical examples of the time course of nitrogen fixation in co-cultures. Nitrogen fixation measured by the acetylene reduction assay was significantly decreased in the *pilAB* mutant. The total amount of ethylene accumulated (µmol) was 17.3 ± 6.6 (a) for BH72, 6.6 ± 2.8 (b) for transposon mutant BH1599 and 16.8 ± 9.1 (a) for the complementation mutant BH1599*pilAB*. Values with standard deviations represent eight cultures each, and are followed by a different letter when significantly different ($P < 0.05$). Nitrogen fixation in pure cultures of mutant BH1599 was not affected in comparison with the wild-type *Azoarcus*. Generation times on N_2 in an oxygen-controlled bioreactor (dissolved O_2 concentrations 0.4–1.2 µM) on SM medium without combined nitrogen were 2.3 h for both strains.

In parallel, the ability of mutants to differentiate into diazosome-containing cells in association with the fungus was evaluated by transmission electron microscopy of ultrathin sections. Intracytoplasmic membranes were visible in wild-type strain BH72 and in the complementation mutant, but not in the *pilAB* mutant, where more than 120 cells were inspected.

Type IV pilin is involved in bacterial colonization of plant roots

Points of emergence of lateral roots and root tips are the preferred sites of grass root colonization and invasion for *Azoarcus* sp. BH72 (Hurek *et al*., 1994a; Reinhold-Hurek and Hurek, 1998a). Therefore, we determined the capacity of *pil* mutants to establish microcolonies on roots of young rice seedlings. Mutants differed significantly from wild-type *Azoarcus* in their colonization capacities. Viable cells of single or double *pilAB* mutants were rarely detected on the surface of main roots and were rarely found to colonize achsils of lateral roots (Fig. 7A–C). Plant cells of the root epidermis were rarely seen to be infected by mutants, in contrast to wild-type bacteria (Fig. 7D and E). The complementation of *pilAB* partially restored the wild-type phenotype: the percentage of lateral root achsils colonized (per main root) ± standard deviation was 29.6 ± 33.6 (a) for strain BH72, 1.3 ± 3.8 (b) for mutant BH1599, 13.7 ± 15.0 (c) for complementation mutant BH1599*pilAB*, and 1.1 ± 2.8 (b) and 1.8 ± 4.1 (b) for complementation mutants BH1599*pilA* and BH1599*pilB* respectively. For each strain, 40–55 main roots of five different plants obtained in two independent experiments were inspected, and numbers followed by different letters are significantly different from each other ($P < 0.05$).

Discussion

Our results suggest that the role of type IV pili in adhesion and infection is not restricted to animal pathogens, but is

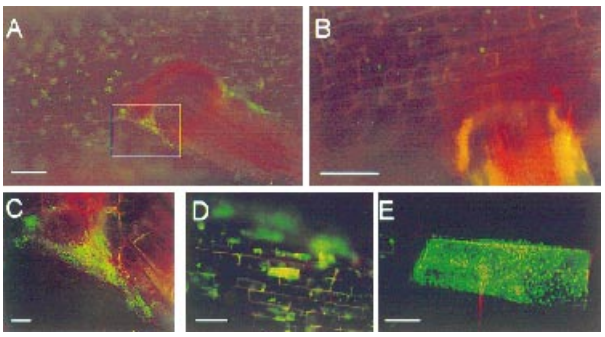

Fig. 7. Colonization of rice roots by *Azoarcus* sp. BH72 and pilin mutants. Rice roots of rice seedlings grown aseptically for 3 weeks in the presence of bacteria were stained for bacterial colonization. Viable bacteria and plant cell nuclei show green fluorescence in fluorescence micrographs. Main root with emerging lateral root inoculated with wild-type *Azoarcus* (A) or transposon mutant BH1599 (B) showing dense or no colonization, respectively.
C. Close-up of boxed area in (A), showing single fluorescing bacteria in the achsil of the lateral root.
D and E. Invasion of a root epidermis cell by wild-type *Azoarcus*.
E. Close-up of (D) showing tightly packed bacteria inside a plant cell. Bars = 50 μm (A, B and D) or 10 μm (C and E).

also a common trait in plant–microbe and fungus–microbe interactions. In the diazotroph *Azoarcus* sp. BH72, an unusually short pilin, PilA, is essential for pilus formation and affects the colonization of plant root and fungal surfaces.

With 59 amino acids, PilA is extremely short compared with other pilin precursors of approximately 160 amino acids (Strom and Lory, 1993). Truncated pilins may also occur in *N. gonorrhoeae*. However, these strains are non-piliated unless they possess an additional, complete gene (Bergström *et al*., 1986). Therefore, it is highly unusual that such a short pilin may form a functional pilus. As (i) Southern hybridization revealed no additional copies of identical genes in the *Azoarcus* genome and (ii) *pilA* mutants lack pili, this 'truncated' pilin appears to be essential for pilus formation in *Azoarcus*. It has been recognized recently that pilins may also play a role in protein excretion as a component of the secretion apparatus for exoenzymes, as proposed for *P. aeruginosa* (Lu *et al*., 1997). This may be the case also for PilA, as we observed a decreased cellulase excretion in the *Azoarcus pilA* mutant (unpublished data).

Pilus formation can also be influenced by mutations in pilus assembly proteins. A complex machinery depending on at least 30 genes is required for the biogenesis and function of type IV pili (Alm and Mattick, 1997). Pilus biogenesis and extracellular protein secretion appear to be evolutionarily related processes. This is reflected in the sequence similarities of some proteins involved in pilus assembly (Russell and Darzins, 1994; Alm and Mattick, 1996; Alm *et al*., 1996) and in the type II secretion pathway (Bally *et al*., 1992; Bleves *et al*., 1998). Several of these proteins share homology with the highly conserved N-terminal region of the type IV prepilin, including the short leader sequence, which is processed by the signal peptidase PilD. The protein encoded by *pilA* of *Azoarcus* sp. BH72 contains the highly conserved G-1F + 1/E + 5 motif that is required for cleavage of the 6- to 7-amino-acid leader sequence and the subsequent methylation of the mature pilin (Strom *et al*., 1993). In a stretch of 25 amino acids, *pilA* showed 100% identity to the conserved region of prepilin of *P. aeruginosa* and only limited homology to pilus assembly proteins (PilE, PilW, PilV, FimT and FimU) or secretory apparatus components (XcpT, XcpU, XcpV and XcpW). Also, most of these proteins are in the range of 15–18 kDa (Alm *et al*., 1996) and therefore not similar to PilA in size. This suggests that PilA is indeed a prepilin and not primarily a part of a pilus assembly or secretion apparatus. Moreover, mutations in proteins of the general secretion pathway are not likely to result in a non-piliated phenotype, as *pilA* does. If PilA was an unusual type IV pilus assembly protein, this would still indicate that type IV pili are involved in the phenotype we observed.

The inner layer of *N. gonorrhoeae* pili is a parallel coiled-coil made of α-helices, which are formed by the 54 N-terminal amino acids, hydrophobic interactions giving stability and flexibility to the fibre (Parge *et al*., 1995; Forest and Tainer, 1997). For the corresponding 53 amino acids of *Azoarcus* PilA, an α-helix was predicted by different secondary structure prediction algorithms, and they might therefore form a similar core of a fibre. In this case, the C-terminal amino acids might be exposed to the entire surface of the pilus, in contrast to the corresponding amino acids 49–56, which appear to be exposed only at the end of the *N. gonorrhoeae* pilus fibre (Forest and Tainer, 1997). However, it is not clear whether, in *Azoarcus*, additional proteins are linked to PilA as a coat of the pilus, especially as the pilus appears to be only slightly smaller in diameter than usual type IV pili. Also, it is not clear how attachment to both plant and fungal surfaces can be mediated, as the *Azoarcus* pilin is lacking domains that are antigenic determinants and involved in receptor recognition or antigenic variation in pathogens (Seifert, 1996). It can be speculated that, as proposed in *Neisseria* (Rudel *et al*., 1995), additional proteins might serve as adhesins in *Azoarcus*, which could mediate the specificity of binding to different plant or fungal surface components. In this context, it is tempting to speculate on the role of *pilB*, which is co-transcribed with *pilA* and essential for pilus formation. PilB and FimF of *E. coli* both have an N-terminal signal sequence typical of secreted proteins and share some, albeit weak, amino acid similarity throughout the sequence (Fig. 2A). FimF is likely to be a minor component of the mature type I fimbriae and also of the assembly apparatus in a FimC–FimF pilus–chaperone complex (Jones *et al*., 1995). As PilB appears to be extruded from

intact cells, according to immunological analyses, it may be speculated that it might have a similar role in biogenesis or function of pili in *Azoarcus* sp. BH72, albeit for type IV and not type I pili. As there is no high homology to proteins known to participate in type IV pilus biogenesis and PilB was found to be essential for piliation in *Azoarcus* sp. BH72, PilB appears to be a novel protein involved in this process. It appears to be sufficient to support pilus production, albeit inefficiently, even when PilA is truncated: in mutant BH1599, in which PilA is shortened by 7 terminal amino acids and *pilB* is disrupted as a result of transposon insertion, complementation with PilB leads to the production of fragments of pili at a very low rate.

It has been recognized recently that surface appendages play a role in plant–pathogen interactions. Plant tumour induction by *Agrobacterium tumefaciens* may depend on a pilus structure (Fullner *et al*., 1996), and a new type of pilus (Hrp pilus) of *Pseudomonas syringae* is involved in eliciting the hypersensitive response of plants (Roine *et al*., 1997). However, this is the first report that type IV pili are involved in plant–microbe and fungus–bacteria interactions. In the plant pathogen *Xanthomonas campestris*, a type IV pilin gene has been detected but has not been observed to have an influence on virulence (Ojanen-Reuhs *et al*., 1997). In contrast, the *Azoarcus* sp. BH72 type IV pilin appears to be crucial for the establishment of microcolonies on grass roots. As colonization of roots by bacteria is a common but poorly understood phenomenon, the detection of genes involved in attachment to roots is an important step towards elucidating this process. As these genes also mediated bacterial adhesion to the mycelium of a rhizosphere fungus, type IV pili appear to have a more general role in the interaction of *Azoarcus* with eukaryotes of its habitat. Non-piliated mutants failed to form diazosomes in co-culture with the fungus, suggesting that spatial proximity to the mycelium is a prerequisite for the formation of these membranes related to efficient nitrogen fixation. One possible reason for this might be the lower oxygen concentrations likely to prevail in niches of the actively respiring mycelium.

Our findings suggest that there are common traits in animal–pathogen and other eukaryote–microbe interactions. Comparative studies of both processes might give important insights into the evolution of pilus-mediated interactions.

Experimental procedures

Bacterial strains and plasmids

Bacterial strains and plasmids are listed in Table 1 (Hanahan, 1983; Yanisch-Perron *et al*., 1985; Reinhold *et al*., 1986; Meissner *et al*., 1987). The asexual ascomycete 2003 was isolated from Kallar grass (Hurek *et al*., 1995) and is affiliated to the *Hypocreales* within the order *Euascomycetes* (Hurek and Reinhold-Hurek, 1998). The isogenic mutant BH1599 had been obtained by mutagenesis with a Tn*5*-derived transposon, Tn*5-gusA2* (Sharma and Signer, 1990), which was kindly supplied by E. Signer. Out of approximately 400 transposon mutants, which were initially screened for loss of endoglucanase activity, one mutant (BH1599) showed a decrease in cellulase activity in plate assays.

Growth conditions

Azoarcus sp. was grown routinely at 37°C on SM medium for nitrogen fixation (Reinhold *et al*., 1986), on VM–ethanol medium (Karg and Reinhold-Hurek, 1996) or on VM medium with potassium malate instead of ethanol for electroporation. Antibiotics (Km and Ap) were added at 30 μg ml^{-1} for mutants. Fungal–bacterial co-cultures were cultivated and evaluated as described previously (Hurek *et al*., 1995; Karg and Reinhold-Hurek, 1996). *E. coli* was grown under standard conditions (Ausubel *et al*., 1987).

Cultivation of plants

Rice seeds (*Oryza sativa* IR36) were surface sterilized and inoculated as described previously (Hurek *et al*., 1994a). However, plants were grown in a 16 h day/8 h night cycle in a greenhouse, and the following plant medium was used (per litre): DL-malic acid, 0.2 g, titrated with KOH to pH 6.8; L-proline, 0.58 g; KH_2PO_4, 1.8 g; KH_2PO_4, 1.2 g; ferric citrate, 13 mg; NaCl, 0.1 g; $CaCl_2$, 26.4 mg; $MgSO_4 \times 7H_2O$, 0.2 g; $Na_2MoO_4 \times 2H_2O$, 4 mg; H_3BO_3, 3 mg; $MnSO_4 \times H_2O$, 12 mg, $ZnSO_4 \times 7H_2O$, 0.2 mg; $CuSO_4 \times 5H_2O$, 0.1 mg; agar, 4 g. After 3 weeks, rice roots were rinsed with water and stained overnight at 4°C with the LIVE/DEAD BacLight bacterial viability kit (Molecular Probes), and colonization was inspected by fluorescence microscopy (see below).

DNA and RNA analyses

Isolation of chromosomal DNA (Hurek *et al*., 1993) and oligonucleotide hybridization (Karg and Reinhold-Hurek, 1996) were carried out as described previously. Other DNA and RNA techniques followed standard protocols (Ausubel *et al*., 1987). Genomic clones were characterized by restriction map and Southern blot analyses. DNA was sequenced from both strands (Hurek *et al*., 1997a). Sequences were analysed and aligned by the CLUSTAL method (Higgins and Sharp, 1989) in the DNASTAR software package. Homology searches were carried out using the BLAST program (Altschul *et al*., 1990). Secondary structure was predicted using the PhD server (Rost *et al*., 1994) or the Nearest Neighbor Secondary Structure Prediction algorithm.

Construction of plasmids for complementation assays and mutant strains

Complementation with *pilAB* and with *pilA* or *pilB* separately was achieved by transformation of the transposon mutant strain BH1599 with the plasmids pJBLP1, pJBLP11 or pJBLP13, respectively, by electroporation and subsequent integration

of the plasmids into the chromosome by homologous recombination. Plasmids were constructed as follows. The insert of pJBLP1 (Fig. 1A) containing *pilAB* originates from pJBL1, which was obtained from a λ-ZAP-Express library from partial *Sau*IIIAI digests of chromosomal DNA of strain BH72. The *pilB* gene of pJBLP1 was inactivated by cloning a Km-resistance cartridge (Pharmacia) into the *Mfe*I restriction site of pJBLP1, resulting in pJBLP11. In frame deletion of *pilA* was achieved by polymerase chain reaction (PCR) using a primer spanning only the first and the last 12 bp of *pilA* (nucleotides 161–201 and 355–402; Fig. 1B). The PCR product containing the *Eco*47III restriction site downstream of the *pilA* deletion was cloned, the correct deletion controlled by sequencing, and the part of pJBLP1 located downstream of the *Eco*47III site was cloned behind the *pilA* deletion, resulting in pJBLP13 (Fig. 1A).

Immunological techniques

Rabbit polyclonal antibodies to PilA or PilB were raised against custom-synthesized peptides (amino acids 29–54 of PilA or amino acids 128–142 of PilB in Fig. 1B) (Eurogentec). Proteins were separated by SDS–PAGE, electroeluted on nitrocellulose and detected on Western blots as outlined previously (Hurek *et al*., 1994a) or by using a chemiluminescence reaction (ECL Western blotting detection reagents; Amersham). To obtain proteins of cell appendages, cells were passed five times through a hypodermic needle, and proteins of the supernatant were precipitated with trichloroacetic acid.

Microscopy

For fluorescence microscopy, bacteria grown on agar plates were transferred to glass slides silanized with 0.185% Bind-Silane (Pharmacia) and 0.185% acetic acid in ethanol. Bacteria were incubated with antibody solutions diluted as follows: anti-PilB, 1:500; swine anti-rabbit fluorescein isothiocyanate (FITC)-conjugated antibody (Dako), 1:30. Before and after antibody incubations, bacteria were washed with PBS.

To visualize fluorescence, bacteria and plant samples were inspected as described previously (Egener *et al*., 1998). Micrographs were recorded by a 3 Chip RGB colour camera (Hamamatsu Photonic Systems). To reduce haze, digital confocal imaging was achieved using Improvision Openlab software (Egener *et al*., 1998).

For transmission electron microscopy, bacteria were grown on agar plates, resuspended in PBS, transferred to charged, Formvar-/carbon-coated copper grids, washed with PBS, fixed with 0.5% glutaraldehyde and negatively stained with 2% neutralized phosphotungstic acid. To visualize intracytoplasmic membranes, cells were embedded in resin, sectioned and stained according to Hurek *et al*. (1994a).

Statistical analysis

The GraphPad InStat software package was used for statistical analysis by ANOVA followed by the Tukey–Kramer test.

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