Flagella Mediate Endophytic Competence Rather Than Act as MAMPS in Rice–*Azoarcus* sp. Strain BH72 Interactions

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***Azoarcus* sp. strain BH72 is an endophytic betaproteobacterium able to colonize rice roots without induction of visible disease symptoms. BH72 possesses one polar flagellum. The genome harbors three copies of putative *fliC* genes, generally encoding the major structural protein flagellin. It is not clear whether, in endophytic interactions, flagella mediate endophytic competence or act as MAMPS (microbe-associated molecular patterns) inducing plant defense responses. Therefore, possible functions of the three *FliC* proteins were investigated. Only *fliC3* was found to be highly expressed in pure culture and in association with rice roots and to be required for bacterial motility, suggesting that it encodes the major flagellin. Endophytic colonization of rice roots was significantly reduced in the in-frame deletion mutant, while the establishment of microcolonies on the root surface was not affected. Moreover, an elicitation of defense responses related to *FliC3* was not observed. In conclusion, our data support the hypothesis that *FliC3* does not play a major role as a MAMP but is required for endophytic colonization in the *Azoarcus*-rice interaction, most likely for spreading inside the plant.**

Azoarcus sp. strain BH72 is a diazotrophic model endophyte of grasses (Hurek and Reinhold-Hurek 2003). The gram-negative bacterium supplies fixed nitrogen (Hurek et al. 2002) to its original host, Kallar grass. In gnotobiotic systems, it is also able to invade rice roots and spread systemically into shoots (Hurek et al. 1994) and to express nitrogenase genes endophytically (Egener et al. 1999). Endophytic rice colonization by *Azoarcus* sp. appears to be an active process, for which several determinants have been detected, such as type IV pili (Dörr et al. 1998), pilus-mediated twitching motility (Böhm et al. 2007), or an endoglucanase (Reinhold-Hurek et al. 2006). Nevertheless, molecular mechanisms of endophyte-plant interactions are not yet well understood. Particularly interesting is how high colonization densities in roots can be achieved without elicitation of plant disease or stress symptoms (Reinhold-Hurek and Hurek 2011). Com-

parative genome analysis suggests that *Azoarcus* sp. strain BH72 might be regarded as a “disarmed pathogen,” lacking genes for types III and IV secretion systems or obvious toxins (Krause et al. 2006). However, for endophytic interactions, the role of other bacterial components that might elicit plant defense responses is not clear.

Defense responses have been well summarized in the so-called zigzag scheme describing a dialog between plant and pathogen (Jones and Dangl 2006). Conserved molecular characteristics of the pathogen (pathogen-associated molecular patterns) or microorganism (microbe-associated molecular patterns [MAMPS]) are recognized by the plant, inducing reactions such as ethylene production, alkalization, or evolution of reactive oxygen species (ROS) (Felix et al. 1999). Fast innate immune responses may be followed by inhibition of the defense mechanisms due to effector proteins of the pathogen.

Among the MAMPS eliciting plant defense responses are bacterial flagella (Medzhitov and Janeway 2002), particularly a conserved peptide of flagellin (*flg22*) (Felix et al. 1999), which is perceived by the receptor-like kinase (RLK) FLS2 in *Arabidopsis thaliana* (Gomez-Gomez and Bolter 2000). Although *flg22* did not elicit detectable responses (Felix et al. 1999) in suspension cultures of rice, flagella from the incompatible *Pseudomonas avenae* (reclassified *Acidovorax avenae*) N1141 induced immune responses on rice (Che et al. 2000). Moreover, 46 rice genes were found to be differentially expressed as part of an immune response mediated by flagellin perception of an incompatible *Acidovorax avenae* strain (Fujiwara et al. 2004).

In endophytic interactions, it has been postulated that lack of flagella might decrease the defense response and, thereby, improve endophytic colonization, which has indeed been observed for a *Salmonella* mutant in *Medicago sativa*, a heterologous system (Iniguez et al. 2005). Contradictory results were reported for nonmotile mutants of *Salmonella enterica* that either lacked flagella or had inactive flagella; the mutants showed decreased invasiveness or spreading in an *Arabidopsis* model (Cooley et al. 2003). Importance of flagella was also shown for competitive colonization of *Medicago truncatula* roots by the rhizoplane colonizer *Pseudomonas fluorescens* F113 (Capdevila et al. 2004). Therefore, it is not yet clear whether flagella mediate endophytic competence or act as MAMPS. In this context, the endophyte *Azoarcus* sp. strain BH72 is equipped with a single polar flagellum (Reinhold-Hurek et al. 1993). Its genome harbors three copies of *fliC* (Krause et al. 2006) commonly encoding the structural protein of flagella, flagellin.

Here, we analyze the role of flagellins for motility of *Azoarcus* sp. strain BH72 and for the endophytic colonization of rice. We conclude that flagella mediate endophytic competence rather than act as MAMPS in this interaction.

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RESULTS

Genome-based analysis

Azoarcus sp. strain BH72 flagellin genes.

Annotation of the *Azoarcus* sp. strain BH72 genome sequence revealed that 48 genes, which are located in three distinct regions, show similarity to genes described to code for proteins involved in flagellum aperture, gene regulation, or assembly of flagellum compounds (Krause et al. 2006). Among them are three copies of *fliC*, commonly encoding flagellin, the structural protein of flagella. They are located in distinct

Fig. 1. Genome structure and protein sequence of *fliC* genes of *Azoarcus* sp. strain BH72. **A**, The genomic regions of *fliC1* (azo2534), *fliC2* (azo2693), and *fliC3* (azo2704), respectively. Genes designated with names comprise open reading frames (ORF) described to code for structural or regulatory components of the flagellar system: *h*, encoding hypothetical protein; upstream of *fliC2*, two ORF encoding GGDEF family proteins; downstream of *ycgR*, an ORF encoding a GGDEF/PAS/PAC domain-containing protein. **B**, Protein domains, predicted by the Pfam database, based on the deduced amino-acid sequence and a cut-off value for domain prediction of $E < 10^{-8}$. Diagonal stripes label bacterial flagellin N-terminus domains (PF00669) (not entirely predicted for FliC1), vertical stripes correspond to bacterial flagellin C-terminus domains (PF00700), and horizontal stripes highlight flagellin hook IN motif (PF07196). **C**, Comparison of *Azoarcus* sp. strain BH72 flagellin sequences, with corresponding sequences of other virulent, plant-pathogenic or plant-beneficial bacteria. A = *Agrobacterium*; S = *Sinorhizobium*; L = *Legionella*; E = *Escherichia*; P = *Pseudomonas*, and X = *Xanthomonas*.

genomic regions, *fliC1* being located approximately 168 kbp upstream of *fliC2* and *fliC3* approximately 16 kbp downstream of *fliC2*. Only *fliC3* is located in one of the three clusters mentioned above, as the first gene of a putative operon (Fig. 1A) including *fliG*, *fliD*, *fliS*, and *fliT* that are all likely to be associated with flagella-driven motility. According to protein domain prediction (The Sanger Institute's Pfam database) the 560, 493, and 493-aa-long proteins FliC1, FliC2, and FliC3, respectively, comprise N- and C-terminal flagellin domains, although the N-terminal flagellin domain may not be complete in FliC1 (Fig. 1B). FliC1 of *Azoarcus* sp. strain BH72 exhibits a relatively low amino-acid identity of 38% with FliC2 or FliC3, while FliC2 shares 80% identity with FliC3. In contrast to the other two flagellins, FliC3 additionally exhibits a flagellin hook IN motif with high probability (Fig. 1B), which is related to a domain with unknown function present in many flagellar hook proteins, including FliD homologs.

FliC1, FliC2, and FliC3 were identical in the 22 amino-acid residues of flagellin 22 (flg22) particularly important for defense elicitation in *Arabidopsis* (Fig. 1C) and shared with pathogens *Legionella pneumophila*, *Xanthomonas campestris*, and *P. avenae* (reclassified *Acidovorax avenae*). The flagellin peptides of the plant pathogen *Agrobacterium tumefaciens* and the plant symbiont *Sinorhizobium meliloti* differ and were shown not to have an alkalization-inducing activity (Felix et al. 1999).

Azoarcus sp. strain BH72 flagellin gene expression analysis.

To study the role of FliC in the endophyte *Azoarcus* sp. strain BH72, plasmid insertion mutants were generated that led to a polar mutation due to a transcriptional terminator sequence. Simultaneously, transcriptional fusions with the reporter genes *gusA* and *gfp* were introduced. Based on the β -glucuronidase activity the expression level of *fliC1*, *fliC2*, or *fliC3* was estimated in exponential and stationary liquid culture (Vollmedium [VM]-ethanol). Expression levels of both *fliC1* and *fliC2* were low in exponential as well as stationary-growth phases, respectively (*fliC1::gusA*, 185 ± 6 or 256 ± 13 Miller units, respectively; *fliC2::gusA*, 408 ± 16 or 511 ± 6 Miller units, respectively). Expression levels of *fliC3* were approximately 20-fold higher than for the other *fliC* genes in both growth phases (*fliC3::gusA*, $7,897 \pm 434$ or $6,690 \pm 620$ Miller units, respectively), indicating that it encoded the major flagellin (data from two independent experiments, each containing six measurements).

In order to test whether gene expression of the other two *fliC* genes was up-regulated in association with rice roots, we made use of the tandem transcriptional fusions of *gfp* and *gusA* in the above-mentioned mutants to analyze green fluorescent protein (GFP) fluorescence. In short-term invasion assays, rice seedlings were grown in the presence of the respective reporter strains for 3 days, and fluorescence of planktonic and root-associated bacterial cells was compared with cells in pure culture (Fig. 2). Single-cell fluorescence indicative of high expression levels (Egener et al. 1998) was neither detected in pure culture of reporter strains *fliC1::gfp* and *fliC2::gfp* as expected nor in planktonic cells incubated with rice roots (Fig. 2, part I). Likewise, cells attached to rice roots did not show fluorescence (Fig. 2, part II, A to D) nor were plant cells detected that were invaded by fluorescent bacterial cells (not shown). In contrast, fluorescence of strain *fliC3::gfp* was readily visible in pure culture, in planktonic cells (Fig. 2, part I), in cells attached to rice roots (Fig. 2, part II, E and F), and in typical invasion zones at the emergence points of lateral roots as well as in epidermal root cells (Fig. 2, part II, G and H). Thus, the two alternative flagellin genes were not likely to be upregulated in the response to the plant to similar levels as *fliC3* was expressed.

Observations were similar for long-term incubation experiments (14 days, not shown).

Flagella dependent motility is impaired in BHfliC3 and BHΔfliC3.

According to genome localization and gene expression levels, we suspected that FliC3 might be the functional flagellin in *Azoarcus* sp. strain BH72, albeit its function might be complemented by FliC1 and FliC2. As *fliC3* is located in a putative operon upstream of genes putatively required for the flagellar apparatus (Fig. 1A), in the insertion mutant BHfliC3, inactivation of the operon by a polar effect is likely. Thus, a *fliC3* in-frame deletion mutant was constructed to avoid such polar effects (BHΔfliC3). In addition to confirmation of the deletion by polymerase chain reaction (PCR) and Southern blot analyses, it was corroborated that, in both deletion mutant BHΔfliC3 and insertion mutant BHfliC3, the FliC3 protein was absent from crude flagella extracts obtained after shearing

and ultracentrifugation (Fig. 3A). Although minor proteins of around 50 kDa in size were still present in the extracts, the major protein seen in the wild-type extract was lacking. Mass spectrometric analysis (matrix-assisted laser desorption-ionization time of flight [MALDI-TOF]) of the putative FliC3 protein in the flagellar preparation from wild type (framed in

Fig. 2. Expression of transcriptional *fliC::gfp* fusions in *Azoarcus* sp. strain BH72 plasmid integration mutants BHfliC1, BHfliC2, and BHfliC3. **I**, Cells from the exponential growth phase in pure culture or in association with roots of rice seedlings as planktonic cells. **II**, **A** to **H**, Cells attached to and invading rice roots. Arrowheads point towards bacterial cells. Phase contrast images are shown next to corresponding fluorescence micrographs (I and IIA to F); settings of the video camera for fluorescence micrographs were identical for all mutants. Images A, C, and E are phase contrast images; images B, D, F, and H are merged micrographs taken with green fluorescence protein filter set 9 and red fluorescence filter set 43HE to demonstrate plant autofluorescence, while image G was obtained with filter set 9 only and low additional phase-contrast illumination. In I, bars = 4.5 μ m; in IIA to F and H, bars = 29 μ m or IIG, = 70 μ m.

Fig. 3. Phenotype of putative flagellin mutants. **A**, Crude extracts of flagella filaments of wild-type strain BH72, in-frame deletion mutant BHΔfliC3, and insertion mutant BHfliC3, separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein (36, 12, or 11 μ g) was loaded for BH72, BHΔfliC3, or BHfliC3 extracts, respectively. M = molecular weight marker (Fermentas, St. Leon-Rot, Germany) in kDa. The boxed area is an example of the gel region used for protein analysis. **B**, Transmission electron microscopic analysis of flagella in the wild type (BH72), BHfliC1, BHfliC2, and BHfliC3; for the latter mutant, no flagella were detectable. Arrowheads point towards flagellum filaments. **C**, Swarming capacity of wild-type strain BH72 and mutant strains in soft agar, as indicated by large milky halos (left side). Right side, quantification of swarming zones from two independent experiments, each with at least four measurements per strain. Bars represent standard deviation. Applying the Tukey-Kramer multiple comparison test, the difference of colony diameter is not significant ($P > 0.05$) for columns with same letter code and is statistically significant ($P < 0.001$) in columns with different letter codes. **D**, Twitching motility observed for the wild type, BHfliC1, BHfliC2, BHfliC3, and BHΔfliC3. Whole colonies showing twitching (upper panel) and close-up of colony boundary with twitching zones (lower panel) are shown. Arrows point to the twitching fringe. Size bars in B = 500 nm and in C = 1 mm or 100 μ m in upper or lower images, respectively.

Fig. 4. Effect of *fliC3* mutation on plant-microbe interactions. **A**, Endophytic colonization of roots of rice seedlings by *Azoarcus* sp. strain BH72 reporter strain BHNG3.1 (wild type carrying *nifH::gusA* reporter gene fusion) and in-frame deletion mutant strain BHΔfliC3, as bacterial life counts in colony forming units (CFU) reisolated from root tissue 14 days postinoculation. Data shown with standard deviations from three independent experiments with eight plants each. The bar labeled with an asterisk is significantly different ($P = 0.0383$), according to paired *t*-test. **B**, Surface colonization of roots of rice seedlings by strain BHNG3.1 and mutant BHΔfliC3, as percent of emergence points of lateral roots containing microcolonies visible by fluorescence microscopy. Data shown with standard deviations from 16 to 19 plants each, not statistically significant according to *t*-test ($P \geq 0.05$). **C**, Crude flagella extracts and **D**, flagellin or flagellin-sized proteins purified by electroelution after separation on sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *Azoarcus* sp. strain BH72 and *Azoarcus* BHΔfliC3 tested for elicitation of ethylene production in *Oryza sativa* OC156 cell culture. Data from the means of three (C) or two (D) independent experiments with three measurements each. Bars labeled with an asterisk represent data that are significantly different ($P < 0.05$) from the negative control, according to paired *t*-test. Positive controls were 150 μ g of pen or 300 ng of chitin. Crude flagellar extracts applied for data shown in C were estimated to be 36 μ g for BH72 or 12 μ g for BHfliC3 and BHΔfliC3, respectively. Approximately 5 μ g of flagellin protein purified from BH72 was applied to obtain data shown in D, with flagellin-sized protein of BHΔfliC3 obtained from corresponding gel slice.

Figure 3A) revealed that, indeed, only FliC3 was detectable, while FliC1 or FliC2 fragments were not detected. Additionally, in extracts of mutants BHfliC1 and BHfliC2, a prominent 50-kDa protein was present, as in wild-type extracts (data not shown). The results suggested that FliC3 was the major compound of *Azoarcus* sp. strain BH72 flagellum filaments.

Electron microscopic inspection of wild-type and mutant cells supported this finding. Flagella were visible for wild-type, BHfliC1-, and BHfliC2-mutant cells but completely absent in mutant BHfliC3 (Fig. 3B). Light microscopic inspection of life cells from liquid medium revealed that both mutants, BHfliC3 and BHΔfliC3, were nonmotile in contrast to the wild-type BH72 and the mutants BHfliC1 and BHfliC2 (data not shown). Furthermore, swarming motility in soft agar, which has been related to the presence of flagella on the cell surface (Harshey 1994), was impaired to the same extent in BHfliC3 and BHΔfliC3 (Fig. 3C). The diameter of swarm colonies was significantly smaller for BHfliC3 and BHΔfliC3 than for wild type, BHfliC1, and BHfliC2 (Fig. 3C). In addition to flagella, type IV pili-mediated twitching motility may be important for swarming (Mattick 2002) and occurs also in *Azoarcus* sp. strain BH72 (Böhm et al. 2007). To exclude the possibility that absence of the flagellum may interfere with type IV pili biogenesis, twitching motility was examined. Twitching motility was unrestricted for all *fliC* mutant strains under investigation, since lacerated outer colony edges characteristic for twitching motility (Darzins 1993) were visible (Fig. 3D). Therefore, a strong influence of the lack of flagella on the ability to develop functional type IV pili can be excluded.

Flagella affect rice root colonization.

While flagella are thought to function as MAMPs eliciting plant defense responses, flagella-dependent motility or attachment could, in contrast, be a premise for plant invasion. Thus, we analyzed whether the bacterial establishment in and on rice roots was affected in the *Azoarcus* mutants. Colonization of the interior of rice roots was not completely abolished in the absence of flagella in strain BHΔfliC3. However the maximum capacity reached by the control reporter strain BHNG3.1, representing wild-type properties, was not achieved (Fig. 4A). Culturable endophytic bacterial cells were significantly reduced by a factor of three in the BHΔfliC3 mutant in comparison with the wild type (3.1×10^6 or 9.5×10^6 per gram of root, respectively). For mutants BHfliC1 or BHfliC2, a statistically significant difference in endophytic colonization in comparison to wild type could not be detected (data not shown). An involvement of flagella in establishment of microcolonies on the root surface at the major entry sites, the emergence points of lateral roots (Hurek et al. 1994; Reinhold-Hurek and Hurek 1998), was also investigated. Colonization appeared slightly but not significantly decreased in BHΔfliC3 compared with the corresponding BHNG3.1 control strain (Fig. 4B) and was not changed in BHfliC1 or BHfliC2 mutants (data not shown).

Plant defense response is not altered in the absence of flagella.

Concerning growth inhibition of rice seedlings by *Azoarcus* sp. strain BH72, no obvious changes in seedling size or root biomass were observed 2 weeks postinoculation with any of the *Azoarcus* mutants under investigation (not shown). As a test for plant defense reactions, ethylene production of the rice cell culture OC156 was analyzed (Felix et al. 1999). Cells challenged with known elicitors, such as penta-*N*-acetyl-chitopen-taose (chitin) (Felix et al. 1999) or an aqueous extract of the mycelium of *Penicillium chrysogenum* (Thuerig et al. 2006),

emitted ethylene as expected (Fig. 4C and D) in comparison to cells treated with buffer, which showed no detectable or very low ethylene production (Fig. 4C and D). Flagellar extracts obtained from wild type led to a reproducible, albeit low, ethylene production (Fig. 4C). A similar ethylene response as to crude flagellar extracts was obtained when bacterial cells or boiled flagella filament extracts were applied (data not shown). Ethylene production was not significantly different in response to extracts containing flagella or not. The in-frame deletion mutant, or BH Δ fliC3, and the insertional mutant BHflic3, in which the flagellar operon containing genes *fliS*, *fliT*, and *fliD* essential for flagellar assembly were inactivated by a polar effect (Fig. 1A), yielded extracts that still stimulated ethylene production (Fig. 4C). Thus, flagellin is not likely to be the sole elicitor responsible for ethylene production, if at all. Since several proteins were coextracted (Fig. 3A), defense elicitation may be attributed to any of these proteins. To further substantiate that flagellin of *Azoarcus* sp. strain BH72 did not elicit a response, flagella filament preparations were purified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and elution of the prominent 50-kDa protein (wild type) or the corresponding gel slice lacking the protein (BH Δ fliC3). As induction of ethylene production was not significantly different from each other and the negative control and was significantly lower than that of the positive control penta-*N*-acetyl-chitopentose (chitin) (Fig. 4D), FliC3 was not likely to be the inducing molecule.

DISCUSSION

The presence of three different *fliC* genes in *Azoarcus* sp. strain BH72 raised questions about which gene encodes the major functional flagellin and whether they might complement each other to achieve motility. A similar gene organization of the *fliC3* locus as in the endophyte strain BH72 was found in the biocontrol strain *P. fluorescens* F113, with *fliC*, *flaG*, *fliD*, *fliS*, *fliT*, and *fleQ* (Capdevila et al. 2004). In contrast to *Azoarcus* sp., the presence of additional *fliC* genes was not described for *P. fluorescens*. More than one flagellin (FlaA, FlaB, FlaC, and FlaD) was found to be encoded in the root nodule symbionts *Rhizobium lupine* H13-3 and *Sinorhizobium meliloti*, in which they build different subunits of a complex flagellar filament, of which FlaA contributes the major compound. Functional flagella can only be assembled if at least one more subunit is present (Scharf et al. 2001). However, genomic organization of *flaA* to *flaD* (Scharf et al. 2001) strongly differs from genome organization of *fliC* genes in *Azoarcus* sp. strain BH72, which are distantly located from each other.

The following line of evidence indicated that FliC3 is the major constituent of flagella in *Azoarcus* sp. and the only FliC protein required for flagellar motility. Transcriptional reporter gene studies showed highest expression levels for *fliC3* in pure culture. Only the *fliC3* mutant was not motile in swarming and microscopic assays and lacked flagella in electron microscopic inspections. Additionally, a 50-kDa protein, which was identi-

Table 1. Strains, plasmids and primers used in this study

Strain, plasmid and primer	Description or primer sequences	Reference
Strains		
<i>Escherichia coli</i>		
DH5 α F'	F' recA1 deoR endA1 hsdR17(rK ⁻ , mK ⁺) phoA supE44 gyrA96 λ -thi-1 relA1 Φ 80dlacZ Δ M15 Δ (lacZYA-argF) _{U169}	Invitrogen, Carlsbad, CA, U.S.A.
<i>Azoarcus</i> sp.		
BH72	Wild type	Reinhold et al. 1986
BHGN3.1	Ap ^r , BH72 containing <i>nifH::gusA</i>	Egener et al. 1999
BHflic1	Km ^r , pK18TF1 integrated in BH72 <i>fliC1</i>	This study
BHflic2	Km ^r , pK18TF2 integrated in BH72 <i>fliC2</i>	This study
BHflic3	Km ^r , pK18TF3 integrated in BH72 <i>fliC3</i>	This study
BH Δ flic3	978 bp in-frame deletion of BH72 <i>fliC3</i>	This study
Plasmids		
pK18mobsacB	Km ^r , RP4 mob region, containing <i>sacB</i> gene	Schäfer et al. 1994
pK18GGST	Km ^r , 163-bp <i>SpeI</i> - <i>PaeI</i> T4-terminator sequence in <i>NheI</i> - <i>PaeI</i> site of pIMGG	Krause et al.2011
pK18TF1	Km ^r , 578-bp <i>HindIII</i> - <i>XbaI</i> fragment of BH72 <i>fliC1</i> in pK18GGST <i>XbaI</i> - <i>HindIII</i> site	This study
pK18TF2	Km ^r , 620-bp <i>HindIII</i> - <i>XbaI</i> fragment of BH72 <i>fliC2</i> in pK18GGST <i>XbaI</i> - <i>HindIII</i> site	This study
pK18TF3	Km ^r , 596-bp <i>HindIII</i> - <i>XbaI</i> fragment of BH72 <i>fliC3</i> in pK18GGST <i>XbaI</i> - <i>HindIII</i> site	This study
pRK2013	Km ^r , tra ⁺ Ap ^r , ColE1 origin, f1 (+) origin, <i>lacZ</i> , P _{lac}	Figurski and Helinski 1979
pPCR-Script Amp SK(+)	Ap ^r , 497 bp <i>fliC3</i> upstream polymerase chain reaction (PCR) fragment in <i>SmaI</i> - <i>ClaI</i> site of pPCR-Script Amp SK(+)	Stratagene, La Jolla, CA, U.S.A.
pPCR-Script Amp SK(+)-up	Ap ^r , 485 bp <i>fliC3</i> downstream PCR fragment in <i>ClaI</i> - <i>Sall</i> site of pPCR-Script Amp SK(+)	This study
pPCR-Script Amp SK(+)-down	Ap ^r , <i>SmaI</i> - <i>ClaI</i> pPCR-Script Amp SK(+)-up fragment in pPCR-Script Amp SK(+)-down	This study
pPCR-Script Amp SK(+)-updown	<i>SmaI</i> - <i>ClaI</i> site	This study
pK18flic3updownmobsac	Km ^r , <i>SmaI</i> - <i>Sall</i> fragment of pPCR-Script Amp SK(+)-updown in pK18mobsacB <i>SmaI</i> - <i>Sall</i> site	This study
Primers		
flic1_for	5'-GCTCTAGATAACGGCCTGCGCATCAATAATTC-3'	This study
flic1_rev	5'-CCCAAGCTTTCGCCATGTCGATGTTGAG-3'	This study
flic2_for	5'-GCTCTAGATAACTCAATGCGTCGCGAGGGTTC-3'	This study
flic2_rev	5'-CCCAAGCTTGGCGCCGTTGATGGTGAC-3'	This study
flic3_for	5'-GCTCTAGATAACTCGCTGCAAGCCTGTCTT-3'	This study
flic3_rev	5'-CCCAAGCTTGGCGAACCCTTGATGGTGAT-3'	This study
flic3down-forward	5'-GCATCGATATGCTGTCGCAAGCCAAC-3'	This study
flic3down-reverse	5'-CTTCGCGATCGAGATGAT-3'	This study
flic3up-forward	5'-GCCCCGGGGTTGCCGAGGTTGGAATG-3'	This study
flic3up-reverseb	5'-GCATCGATGTTGGTGTGATGACTTGTGC-3'	This study

fied as *FliC3* by MALDI-TOF analyses, was lacking in crude extracellular extracts of both *fliC3*-deletion and insertion mutants. However, these data were obtained in pure culture, and variations in flagellum composition may be possible in response to different environmental conditions. Modification of flagella assembly was, for example, observed in *Vibrio parahaemolyticus* and *V. aginolyticus* possessing a polar flagellum in liquid medium and forming lateral flagella under specific conditions, such as solid surfaces (Atsumi et al. 1996; Shinoda and Okamoto 1977). On either liquid or solid media, lateral flagella were never observed for strain BH72 (this study; Reinhold-Hurek et al. 1993). Moreover, in association with rice roots, expression of *fliC1* and *fliC2* was not strongly upregulated, as indicated by *fliC::gfp* reporter strains, which argues against a major role of these flagellins in interaction with the plant. The functions of *FliC1* and *FliC2* in strain BH72 are thus still unclear.

A slight decrease in endophytic rice root colonization capacity of the *fliC3* deletion mutant provided evidence for the importance of flagella during plant invasion by *Azoarcus* sp. strain BH72, while root-surface colonization was hardly affected. Thus, flagella constitute another factor contributing to the ability to colonize plants endophytically in addition to type IV pili (Dörr et al. 1998), the ability to move on surfaces by twitching motility (Böhm et al. 2007), or an endoglucanase (Reinhold-Hurek et al. 2006). Flagellar motility is known to play a role in other systems. *FliC* of *P. fluorescens* F113 was found to be important for motility and competitive colonization of the root surface (Capdevila et al. 2004), and also, *P. fluorescens* WCS374 requires motility for colonization of potato roots. In pathogens, motility was found to play a role for virulence, e.g., in *Erwinia carotovora* subsp. *carotovora* (Hossain et al. 2005) or in *Ralstonia solanacearum* in tomato (Tans-Kersten et al. 2001), if bacteria had to enter through root invasion and were not supplied directly to wounded tissue (Tans-Kersten et al. 2001). No significant effect of the *fliC3* mutation was observed for the establishment of microcolonies of *Azoarcus* sp. on the root surface. In contrast, *Azospirillum brasilense* uses the flagellum for adherence on roots of *Triticum aestivum* (Rodriguez-Navarro et al. 2007). The test system may, of course, affect the result, as shown for *R. solanacearum* (Tans-Kersten et al. 2001). In our gnotobiotic rice-*Azoarcus* culture, bacteria are provided at high titre and are suspended in the entire medium. This might facilitate access to the root surface and mask traits for rhizosphere competence. Competition experiments between wild type and mutant might reveal more subtle phenotypic differences with higher resolution; however, they were not applied because, in competition assays, the effects of MAMPS will be masked as the wild type is present in the same experimental setup. Moreover, for a type IV pilus mutant of *Azoarcus* sp. (Dörr et al. 1998), a strongly impeded microcolony formation was detectable in a single-strain test system similar to our present study. This suggests that flagella play a comparatively minor role for establishment of *Azoarcus* sp. on the root surface.

In accordance with the observation that flagella contribute to endophytic colonization is the apparent lack of elicitation of a defense response in rice by *Azoarcus* flagellin. Ethylene production in rice suspension cultures was not induced by flagellin obtained from SDS-PAGE. Concordantly, crude flagella preparations still triggered ethylene production, even in the flagella-lacking mutants BHΔ*fliC3* and BH*fliC3*. In the plasmid insertion mutant BH*fliC3*, *fliD*, *fliS*, and *fliT* genes are part of the inactivated putative operon. In *Escherichia coli* (Kawagishi et al. 1992) and *Salmonella typhimurium* (Yokoseki et al. 1995), the *fliD*, *fliS*, and *fliT* genes are indispensable for flagellation; therefore, mutant BH*fliC3* probably cannot assemble

flagella even from *FliC1* or *FliC2* proteins and should be devoid of flagellins in flagellar extracts. Which extracellular molecule did elicit the response in these high-molecular-weight preparations that had been obtained by ultracentrifugation is not clear. Besides *flg22* (Felix et al. 1999), other molecules acting as MAMPS are known, such as cold-shock protein (Felix and Boller 2003) or a peptide of elongation factor TU (Kunze et al. 2004). As-yet-unknown molecules are also likely to exist (Pfund et al. 2004; Zipfel 2009).

Rice appears to react to flagellin in a way quite different from dicotyledonous plants. Suspension cultures did not respond to *flg22* or flagellin from *P. syringae*, e.g., with medium alkalization (Felix et al. 1999) or in a more sensitive test system (Takai et al. 2007). Although rice has a *flg22* perception system, *flg22* was only able to induce a weak immune response (Takai et al. 2008). However, for flagella of incompatible *P. avenae* (reclassified as *Acidovorax avenae*) N1141, programmed cell death and the expression of some defense-related genes was observed (Che et al. 2000). *P. avenae* incompatible and compatible strains (strains N1141 and H8301, respectively) showed strain-specific defense elicitation in rice restricted to flagella of the incompatible strain, with the *flg22* region lacking the majority of the elicitor activity (Che et al. 2000). It is, therefore, difficult to predict elicitor activity for the *Azoarcus* flagellin, which shares high similarity to the common *flg22* region, and our results suggest that flagella of *Azoarcus* spp. strain BH72 do not trigger strong defense responses; however, very weak responses that might be detected in a more sensitive test system cannot be excluded. It was suspected that glycosylation of the flagellin might be important for the rice response (Takai et al. 2008), however a transgenically expressed flagellin from the incompatible strain N1141 induced immune responses and disease resistance (Takakura et al. 2008).

In conclusion, the results provide evidence for a role of *FliC3* in building the major subunit of *Azoarcus* sp. strain BH72 flagella, in swarming motility, and in endophytic colonization of rice. Similar to a mutation in *pilT* leading to defects in twitching motility mediated by type IV pili (Böhm et al. 2007), the lack of flagella led to a more pronounced effect in endophytic colonization than in root-surface colonization. This indicates that surface-dependent and swimming motility might be characteristics that are important for spreading and establishment inside the host. At least in the rice-endophyte interaction, flagella appear to mediate endophytic competence rather than act as MAMPS.

MATERIALS AND METHODS

Bacterial strains and plant material.

Strains as well as plasmids and primers used for cloning are listed in Table 1. *Oryza sativa* subsp. *indica* cv. IR36 originated from the International Rice Research Institute, Los Baños, Laguna, Philippines. Rice-cell cultures OC156 were provided by G. Felix, University of Tübingen (The Center for Plant Molecular Biology), Germany.

Media and growth conditions.

E. coli was grown according to standard protocols (Ausubel et al. 1987) in Luria broth (LB) medium, aerobically at 37°C. *Azoarcus* strains were grown aerobically at 37°C in VM-ethanol medium, a complex medium with ethanol as carbon source (Hurek et al. 1995) for all experiments except for rice inoculation, for which main cultures were grown in SM medium supplemented with 0.1 g of yeast extract and 0.5 g of NH_4Cl per liter (Reinhold et al. 1985). Rice-cell culture OC156 was grown aerobically at 23°C under continuous light supplied by an Osram L 58W/827 (30 to 60 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) in me-

dium containing 4.41 g of Murashige and Skoog supplemented with Nitsch vitamin mixture (Duchefa, Haarlem, The Netherlands), 30 g of sucrose, 45.78 mg of 2-(*N*-morpholino)ethanesulfonic acid (MES), and 2 mg of 2,4-dichlorophenoxyacetic acid per liter, pH was adjusted to 5.6 to 5.8. Rice-cell cultures were diluted weekly with fresh medium to provide continuous growth.

Plant inoculation.

For rice inoculation, surface sterilization of dehusked caryopses was performed as described previously (Hurek et al. 1994), with the following minor modifications: washing steps were extended to three times, each for a minimum of 1 h in sterile double distilled water, and germination plates contained 10 g of agar (Difco, Becton and Dickinson Company, Franklin Lakes, NJ, U.S.A.) per liter. Cells from the exponential-growth phase (optical density at 578 nm [OD_{578nm}] = 0.4 to 1.0) were washed in N-free medium, and approximately 2×10^8 cells were applied per plant as described, with one half of the inoculum suspended in the entire plant medium and the other half applied to the top of the medium (Böhm et al. 2007). Plants were grown in plant medium (Hurek et al. 1994) as described previously (Egener et al. 1999) without L-proline and with quartz sand replacing agar. The malate concentration was increased to 20 mg per liter of DL-malic acid, and the phosphate buffer concentration of 0.88 to 1.12 g of K_2HPO_4 per liter at pH 6.8. Following inoculation, rice plants were incubated in a phytotron at 30°C, at 80% humidity and with a 14-h-day and 10-h-night cycle (approximately 100 μ mol photons $m^{-2} s^{-1}$) or in a greenhouse at similar conditions with a 12-h-day and 12-h-night cycle.

Short-term invasion assays for GFP fluorescence were carried out as previously described for 3 days in liquid medium (Reinhold-Hurek et al. 2006), except that the above-mentioned plant medium was supplemented with 200 mg of DL-malic acid (neutralized) per liter.

Mutant construction and DNA manipulations.

Transformation and extraction of plasmid or genomic DNA was performed analogous to standard techniques (Ausubel et al. 1987). Construction of insertion mutant strains BH*fliC1*, BH*fliC2*, and BH*fliC3* were carried out by plasmid integration mutagenesis using vector pK18GGST (Krause et al. 2011), in which PCR-generated fragments of approximately 600 bp in length were inserted. As they represented a fragment close to the 5' end of the target gene, after single homologous recombinations, the gene of interest was interrupted and transcriptionally fused to reporter genes *gfp* (Egener et al. 1998) and *gusA* from *E. coli*, and transcription of the downstream region was inhibited by a transcriptional terminator. For genes *fliC1*, *fliC2*, or *fliC3*, fragments of 578, 620, or 596 bp in length were amplified by PCR, respectively. Based on the restriction sites introduced in primers listed in Table 1 (*XbaI* and *HindIII*) directional cloning into pK18GGST was conducted. The resulting plasmid was transferred by conjugation and integrated into the genome of *Azoarcus* sp. strain BH72 via homologous recombination, as verified by Southern blot analysis.

For the construction of the *fliC3* in-frame deletion mutant BH Δ *fliC3*, PCR fragments were amplified using *Pfu* DNA polymerase from Promega (Madison, WI, U.S.A.). A 497-bp fragment upstream of *fliC3*, including 25 bp of the *fliC3* gene and a 485-bp fragment spanning the 3' end of the *fliC3* gene, were amplified and cloned into pPCR-Script Amp SK+, yielding pPCR-Script Amp SK(+)*up* and pPCR-Script Amp SK(+)*down* (Table 1). Accuracy was confirmed by sequencing inserts using the dideoxynucleotide chain termination method on an ALFexpress automated sequencer (GE Healthcare, Frei-

burg, Germany) (Hurek et al. 1997). Based on the incorporation of restriction sites into the primers used for PCR (*SmaI* and *ClaI* or *ClaI* and *SalI*, respectively), both fragments were joined by subcloning (pPCR-Script Amp SK(+)*updown*) and were then transferred into the mobilizable vector pK18mobsacB (pK18*fliC3updownmobsac*). This suicide vector was transferred to strain BH72 by conjugation and was integrated into the genome of *Azoarcus* sp. strain BH72 via single homologous recombination. Sucrose selection based on the *sacB* gene yielded the double recombinant BH Δ *fliC3*, in which only 78 bp of *fliC3* remain, corresponding to 26 amino acids. Deletion of *fliC3* was confirmed by PCR and Southern blot analysis.

β -Glucuronidase expression assay.

For quantitative measurements of *fliC1*, *fliC2*, and *fliC3* expression, the corresponding *Azoarcus* reporter strains carrying transcriptional fusions (BH*fliC1*, BH*fliC2*, and BH*fliC3*) were grown in liquid medium and β -glucuronidase activity was estimated as described (Egener et al. 1999). One Miller unit of GUS activity was defined as the absorbance at 420 nm per minute times an OD_{600nm} of 1.

Analyses of flagella.

Strains were grown under moist conditions on VM-ethanol agar plates. To separate flagella filaments from cells, suspensions of cells in 50 mM TrisHCl (pH 8.0) were vigorously agitated for 2 min additionally to threefold extrusion through a needle (0.6 mm $\times$ 25 mm). Flagella filaments and cell debris were separated by 10 min of centrifugation at $3,220 \times g$ twice at 4°C, before filaments were pelleted by ultracentrifugation for 30 min at $67,400 \times g$. Resuspension of flagella filaments in 10 mM TrisHCl (pH 7.2) was followed by brief (1 min) centrifugation at $16,100 \times g$, and the supernatant containing extracted flagella filaments was stored at -20°C as crude flagella extract.

Protein separation by SDS-PAGE and Coomassie staining was performed according to standard procedures. For MALDI-TOF analysis, gel slices containing flagellin were washed three times using a 1:1 mixture of 50 mM NH_4NCO_3 and acetonitrile (ACN). After dehydration of gel slices with ACN, proteins were reduced by 10 mM dithiothreitol for 1 h at 56°C. To alkylate resulting reduced sulfhydryl groups, gel slices were exposed to indolacetamide, were incubated in the dark for 45 min, and were again washed three times in 1:1 NH_4NCO_3 and ACN. After dehydration of proteins due to application of ACN, gel slices were dried for trypsin digestion. By sonification of gel slices in ACN, digested protein fragments were released. Proteins in ACN or in 30% ACN with 1% trifluoro acetic acid (TFA) were dried for MALDI-TOF analyses. Trypsin-digested protein fragments in 0.1% TFA were diluted either 1:2 or 1:10 with saturated α -cyano-4-hydroxycinnamic acid as matrix, and dried crystals were cannonaded with 500 laser shots per second, thereby ions were exposed to 20-kV acceleration voltage and TOF was monitored in the reflector modus. Different spectra were merged, were calibrated with external standard 1 and 2 from Applied Biosystems (Langen, Germany), and were analyzed with Dataexplorer-Software (Applied Biosystems) and the peptide search function Mascot.

For transmission electron microscopical inspection of flagella, cells from agar plates were analyzed as described previously (Böhm et al. 2007).

Motility assays.

To analyze swarming motility, precultures were grown overnight in VM-ethanol medium with appropriate antibiotics, cells were pelleted by centrifugation, cell numbers were adjusted to 5×10^8 cells per milliliter, and 15 μ l was applied on semisolid agar plates (agar 3 g/liter). Bacteria were grown for

18 h under humid conditions at 37°C, and colony diameters were determined. For twitching motility assays, bacteria were grown on moist VM-ethanol agar plates, and twitching zones were monitored via binocular microscope (Olympus SZX12) and C5810 Camera (Hamamatsu, Hersching, Germany) (Böhm et al. 2007).

Assessment of root colonization.

Endophytic rice-root colonization was estimated at 13 to 14 days after inoculation as described previously (Böhm et al. 2007). In brief, roots were washed and exposed to 15 min of ultrasonication (Transonic T420 [Elma GmbH, Singen, Germany], with maximum output) for removal of surface bacteria and were ground in medium devoid of a carbon source containing, per liter, 0.2 g of $\text{MgSO}_4 \times 7\text{H}_2\text{O}$, 0.1 g of NaCl, 0.01 g of $\text{MnSO}_4 \times \text{H}_2\text{O}$, 2 mg of $\text{Na}_2\text{MoO}_4 \times \text{H}_2\text{O}$, 0.026 g of $\text{CaCl}_2 \times 2\text{H}_2\text{O}$, 66 mg of Fe(III)-EDTA, and potassium-phosphate buffer with a final concentration of 0.88 to 1.12 g of K_2HPO_4 at pH 6.8. Appropriate dilutions were spread in agar plates containing SM medium supplemented with 0.1 g of yeast extract, 0.5 g of NH_4Cl , and 8 g of agar per liter. The number of colony-forming units (CFU) per milligram of root fresh weight was estimated after growth of bacteria at 37°C.

To analyze the establishment of microcolonies at the root surface 13 to 14 days postinoculation, bacteria on roots were stained with the LIVE/DEAD BacLight bacterial viability kit (Invitrogen, Paisley, U.K.), as described previously (Böhm et al. 2007). Fluorescence was inspected at the emergence points of lateral roots along the main root, employing a fluorescence microscope (Axioplan) with filter set 09 (Carl Zeiss AG, Jena, Germany), which was also used for GFP fluorescence analysis in which images were merged with red fluorescence (filter set 43 HE) to demonstrate autofluorescence of roots.

Tests for plant defense responses.

Ethylene production was determined based on the method described by Felix and associates (1999). Rice-cell culture OC156 was used to test potential elicitors. Each measurement was done in triplicate after a 3 to 4 h of incubation time of elicitor with 500 µl of cell culture incubated at conditions specified above. For ethylene detection, 1 ml of 3.5-ml head-space was measured by gas chromatography (Egener et al. 1999).

Statistical analysis.

Statistical analysis was carried out by paired *t*-test or Tukey-Kramer multiple comparison test in the GraphPad software package (San Diego, CA, U.S.A.).

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The Sanger Institute's Pfam database: pfam.sanger.ac.uk