Exploring the Function of Alcohol Dehydrogenases During the Endophytic Life of *Azoarcus* Sp. Strain BH72

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The endophytic bacterium *Azoarcus* sp. strain BH72 is capable of colonizing the interior of rice roots, where it finds suitable physicochemical properties for multiplying and fixing nitrogen. Because these properties are poorly understood, a microtiter-plate-based screening of a transcriptional *gfp* (green fluorescent protein) fusion library of *Azoarcus* sp. grown under different conditions was performed. Monitoring of the GFP activity allowed the identification of a gene highly expressed in medium supplemented with ethanol. Sequence analysis revealed that this gene encodes a pyrrolo-quinoline quinone-dependent alcohol dehydrogenase (ADH). Inspection of the complete genome sequence of the *Azoarcus* sp. strain BH72 identified seven additional genes encoding putative ADH, indicating that BH72 is well equipped to survive in different environmental conditions offering various alcohols as carbon source. Analyses of these eight putative ADH showed that expression of three was induced by ethanol, of which two were also expressed inside rice roots. The fact that waterlogged plants such as rice accumulate ethanol suggests that ethanol occurs in sufficiently high concentration within the root to induce expression of bacterial ADH. Disruption of these two ADH evoked a reduced competitiveness to the wild type in colonizing rice roots internally. Thus, it is likely that ethanol is an important carbon source for the endophytic life of *Azoarcus* sp.

The nitrogen-fixing bacterium *Azoarcus* sp. strain BH72 is a diazotrophic endophyte isolated from roots of Kallar grass (*Leptochloa fusca* L. Kunth) (Reinhold et al. 1986), a grass highly tolerant to waterlogging and salinity often used in Pakistan as the primary colonizer of salt-affected lands. Additionally, *Azoarcus* sp. is capable of colonizing rice roots under laboratory conditions in a gnotobiotic system (Hurek et al. 1994). Endophytic colonization of roots by *Azoarcus* sp. takes place actively. Entry sites are the zone of elongation above the root tip where the cells are still undifferentiated or the emergence points of lateral roots (Reinhold-Hurek and Hurek

1998). Inside plants, *Azoarcus* sp. is observed mainly apoplastically. Colonized tissues are the outer cell layers and the root cortex, especially the aerenchyma, the main colonization site. Even the central part of roots can be invaded, from where BH72 may spread into the vegetative body of the plant (Hurek et al. 1994). Although root cells are densely colonized, no symptoms of plant disease are visible, and very limited jasmonic acid-induced defense responses are apparent (Miché et al. 2006).

Although the *Azoarcus* genome sequence is available (Krause et al. 2006), little is known about the genetic requirements important for the active colonization of roots. Thus far, only three determinants were identified: the type IV pili, which are important for the initial interaction with the root (Dörr et al. 1998); and the endoglucanase EglA and the PilT-mediated twitching motility, which both contribute to the invasion of the root interior (Böhm et al. 2007; Reinhold-Hurek et al. 2006).

Although *Azoarcus* sp. depends on a strictly respiratory metabolism, the genome carries all genetic information necessary for performing biological nitrogen fixation (Krause et al. 2006), a process requesting high energy supply and low concentration of oxygen and combined nitrogen. High nitrogenase gene expression, the key enzyme for N₂ fixation, was shown to occur at the entry points of colonization plus deep inside in the intercellular space of the cortex (Egener et al. 1998, 1999). This expression pattern indicates that *Azoarcus* sp. finds a suitable environment for reducing atmospheric nitrogen within the plant, most probably supported by apoplastic nutrients (Egener et al. 1999; Reinhold-Hurek and Hurek 1998). Because of the high nitrogenase gene expression, this nutrient pool must be considerable; however, the composition of this pool in grasses is unknown.

Physiological experiments (Reinhold-Hurek et al. 1993a,b) and the *Azoarcus* genome sequence (Krause et al. 2006) revealed that the bacterium is unable to metabolize common carbohydrates and cell wall constituents, compounds expected to be present in the apoplast. This raises the question of which carbon sources this *Azoarcus* sp. can access in order to perform their energy-demanding, nitrogen-fixing endophytic lifestyle. Here, we describe an experimental approach to apply bacterial promoter fusions for probing the root interior for putative carbon sources, which lead to the identification of alcohol as an important carbon source for *Azoarcus* sp. in planta.

RESULTS

Green fluorescent protein-based screening for substrate- and plant-upregulated *Azoarcus* genes.

The experimental approach to probe the apoplastic nutrient pool of rice roots was based on the identification of *Azoarcus* genes specifically expressed in response to the availability of carbon substrates possibly present in the apoplast. Identifica-

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tion was performed by a promoter-trapping strategy which selects for promoters driving expression of marker genes under certain conditions (Cowie et al. 2006), based on the construction of a transcriptional fusion library to *gfp-gusA* encoding green fluorescent protein and β -glucuronidase in *Azoarcus* sp. Briefly, the construction included ligation of random chromosomal DNA fragments into the vector pIMGG carrying the promoterless reporter genes *gfp* and *gusA* (Fig. 1), followed by conjugation of the plasmid library into wild-type *Azoarcus* sp. strain BH72. After selection on the vector-encoded kanamycin-resistant (Km^r) marker, 1.2×10^3 homologous recombinants were obtained. Integration of the recombinant plasmids into the genome of *Azoarcus* circumvents the problem that a plasmidic fusion library counts on the presence of a promoter in the cloned DNA fragment and might disturb regulatory circuits due to copy number effects. The recombinants were thereafter subjected to a microtiter-plate based GFP screening. For this, the recombinants were transferred in parallel to microtiter plates containing media supplemented with 52 different carbon sources (Supplementary Table S1), which can be either utilized by strain BH72 for growth or which are common metabolites. Expression of the marker gene *gfp* was monitored after 4 h of cultivation by scanning for GFP fluorescence, which was subsequently normalized against the cell density and compared with the green fluorescence of cells incubated in carbon-free medium. Detection of green fluorescence was an indicator of induced gene expression and, therefore, represented candidate promoters for probing the apoplasmic nutrient pool.

Fig. 1. Schematic map of the vectors pIMGG and pK18GGST. A cartridge containing, in tandem, the promoterless reporter genes *gfp* and *gusA*, both carrying upstream of the open reading frame stop codons in all three reading frames and a Shine-Dalgarno sequence commonly used in BH72 (Egener et al. 1998, 1999), was cloned into the *SalI-SmaI* sites of the mobilizable suicide vector pK12mob2 (Kirchner and Tauch 2003) generating pIMGG with a multiple cloning site upstream of *gfp* for inserting DNA fragments. By replacing the *NheI-SphI* fragment through a *SpeI-SphI* fragment carrying the T4 transcription-termination sequence of pH45 Ω (Prentki and Krisch 1984) and an additional unique *XbaI* site, vector pK18GGST was created.

Highest fluorescence was observed with one recombinant when ethanol was added to the growth medium, one of the major carbon sources for strain BH72 (Reinhold-Hurek et al. 2005). This observation was reevaluated by monitoring the second reporter protein, GusA, in β -glucuronidase (GUS) activity assays. The results showed an induction of expression of at least seven times when cultures were grown with ethanol compared with those cultivated in malate, a second major carbon source for *Azoarcus* spp. (Reinhold-Hurek et al. 1993b). The sequence of the *gfp* flanking region of the corresponding pIMGG clone was obtained and used for a search against the *Azoarcus* genome (Krause et al. 2006) and protein database. The identified gene was annotated as *exaA3*, a gene encoding a pyrrolo-quinoline quinone (PQQ)-dependent alcohol dehydrogenase (ADH) which is in good agreement with the induction of gene expression by ethanol.

Genome-wide identification of genes encoding ADH.

The *Azoarcus* genome sequence showed that, in addition to *exaA3*, four additional genes encode proteins with high similarity to PQQ-dependent ADH (ExaA1, ExaA2, ExaA4, and ExaA5) (Krause et al. 2006). All five predicted proteins contain structural characteristics of PQQ-dependent ADH: the conserved amino acid residues involved in PQQ binding, the eight so-called W motifs of the anti-parallel β -sheets (Toyama et al. 2004), and an N-terminal signal peptide indicating a periplasmic localization known for these ADH.

On the basis of their molecular and catalytic properties, the PQQ-dependent ADH have been classified into three types (Toyama et al. 2004). In *Azoarcus* sp., two types were found: ExaA2, ExaA3, and ExaA5, belonging to quinoproteins which have only PQQ as prosthetic group and which transfer electrons to a separate soluble cytochrome; and ExaA1 and ExaA4, with the characteristics of quinohaemoproteins having a C-terminal cytochrome domain covalently attached to the ADH polypeptide chain. This grouping is supported by the correlation with highest amino acid sequence identity to each other (ExaA2 to ExaA3, 52.5%; ExaA1 to ExaA4, 46.6%). Only ExaA5 seems to be an exception; also belonging to quinoproteins, it has a sequence identity of approximately 30% to all other PQQ-dependent ADH of *Azoarcus* sp. strain BH72.

A scan of the protein sequences encoded by *Azoarcus* sp. with the help of the Pfam database (Finn et al. 2006) identified additional putative ADH, all belonging to the group of NAD(P)-dependent ADH. One of these ADH, Azo2700, carries the domain for iron-containing ADH (PF00465). Seven proteins had domains (PF00107 and PF08240) usually found in zinc-binding ADH or in NADP-dependent quinone oxidoreductases, but only two of these proteins (AdhA and AdhC) possessed the conserved amino acids which are related to the zinc, alcohol, and coenzyme binding (Reid and Fewson 1994; Sun and Plapp 1992). Furthermore, these two showed high amino acid identity to other well-characterized zinc-binding ADH. Therefore, only AdhA and AdhC were considered to be true ADH while the others were most probably quinone oxidoreductases.

As expected, AdhA and Azo2700 do not carry any predictable signal peptide or transmembrane helices which point to a cytoplasmic localization of the enzymes. Interestingly, for AdhC, two transmembrane helices were predicted between the N-terminal and the C-terminal domain. This indicates an unusual localization in the membrane with an inside-outside-inside orientation.

Not all ADH are important for ethanol utilization.

In general, *Azoarcus* sp. strain BH72 utilizes dicarboxylic acids or ethanol as major carbon sources (Reinhold-Hurek et

al. 1993b). No growth could be observed when other alcohols such as methanol, propanol, or butanol were used. To elucidate the role of the different ADH in the first reaction step of the ethanol oxidation process, mutants of each gene were constructed through integration of the plasmids pIMGG or p18KGGST into the genome (Table 1). Cultivation of these mutants aerobically on ethanol showed significantly reduced growth rates only when *exaA2* and *exaA3* were disrupted (Fig. 2A). Interestingly, proteomic studies of strain BH72 grown under aerobic standard conditions on ethanol revealed only the presence of these two ADH, with ExaA2 being one of the most abundant proteins identified (Hauberg et al. 2010). These results indicate that ExaA2 and ExaA3 are the main ADH in *Azoarcus* sp.

In a nitrogen-free, microaerobic environment, the growth considerably declined for the mutant *BHhexA2S* and *BHhexA3K* but also for *BHadhC* (Fig. 2A), revealing that there are different requirements in ethanol oxidation under different oxygen regimes.

Expression profile of ADH in free-living *Azoarcus* sp.

To study whether expression of *adh* genes is induced by alcohol, the integration mutants mentioned above were used. Addition of ethanol to cultures cultivated aerobically led to an increased expression of *exaA2*, *exaA3*, and *adhC* (Fig. 2B). Inductions of ADH expression varied between factors of three to seven, depending on the gene analyzed, and were even visible at an ethanol concentration as low as 1 mM. In a nitrogen-fixing microaerobic environment, expression of *exaA2*, *exaA3*, and *adhC* were also strongly induced through ethanol addition (Fig. 2B). Moreover, for *exaA2* and *exaA3*, expression was significantly higher under microaerobic versus aerobic conditions (defined by the *t* test, $P < 0.001$), which was also reflected by the more pronounced induction factors of seven and ten, respectively.

When cells were cultivated aerobically or microaerobically in a carbon-free medium, an enhanced expression of *exaA2*, *exaA3*, and *adhC* was also detectable compared with the expression in malate-containing medium, which most probably resulted from starvation (Fig. 2B). Other alcohols such as methanol, propanol, or butanol did not induce expression levels of *exaA2*, *exaA3*, and *adhC* to the same extent as ethanol. The low induction values which were in a range similar to that of carbon-free medium probably reflect a response to starvation, because strain BH72 cannot grow on these alcohols.

To investigate the effect of malate on the ethanol-induced expression of ADH, cells were cultivated in the presence of various malate/ethanol mixtures. Results showed that no induction could be observed when malate was present (Fig. 3), even at concentrations of as little as $1/10$ of the ethanol concentration. Addition of fumarate, α -ketoglutarate, lactate, or pyruvate, all carbon sources metabolized by *Azoarcus* sp. strain BH72 (Reinhold-Hurek et al. 1993b), to an ethanol-containing medium inhibited induction of ADH expression as well (data not shown). These observations imply that induction of ADH expression in *Azoarcus* sp. is subjected to catabolite repression.

ExaA2 and ExaA3 are also expressed during interaction with rice or an ascomycete.

Azoarcus sp. shows dense colonization of the outer cell layers and the cortex of rice roots cultured gnotobiotically (Hurek et al. 1994). Additionally, in co-culture with the fungal endophyte *Acremonium alternatum* 2003, also originating from Kallar grass, most cells of strain BH72 adhere tightly to the mycelium and develop intracytoplasmic membrane stacks to which the nitrogenase localizes (Hurek et al. 1995). During both interactions, strain BH72 fixes nitrogen. Interestingly, Karg and Reinhold-Hurek (1996) observed expression of *exaA3* during co-cultivation with the ascomycete while Reinhold-Hurek and associates (2007) noticed expression of the same gene in rice roots. This raised the question of ADH expression during interaction with these two partners. Analyses of protein profiles obtained from inoculated rice roots or from *Azoarcus*-ascomycete co-cultivations, both cultured in medium without ethanol addition, identified ExaA3 as expected but, in addition, ExaA2 (Fig. 4). None of the other ADH were found.

Expression of ExaA2 and ExaA3 in rice roots was also visualized through the presence of GFP fluorescence obtained after inoculation of the roots with the corresponding mutant strains carrying a transcriptional fusion of the ADH-encoding gene to *gfp* (Fig. 5A and D). Because single-cell fluorescence could only be observed when *Azoarcus* sp. was cultivated in ethanol-containing medium (Fig. 5B and E) compared with growth in medium with malate (Fig. C and F), it can be assumed that ethanol is produced by the eukaryotic partner in sufficiently high concentration to induce expression of these two PQQ-dependent ADH. The *adhC* mutant strain also showed single-cell GFP fluorescence when cultivated in an ethanol-containing medium, but no fluorescence was detectable in rice roots (data not shown), indicating that this ADH does not play an impor-

Table 1. Bacterial strains

Strain	Relevant characteristics	Source, reference
<i>Escherichia coli</i>		
DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> AM15 Δ <i>lacX</i> 74 <i>recA1</i> <i>endA1</i> <i>araD</i> 139 Δ (<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> λ^- <i>rpsL</i> <i>nupG</i> , Sm ^r	Invitrogen, Karlsruhe, Germany
DH5 α F'	F'/ ϕ 80 <i>lacZ</i> AM15 Δ (<i>lacZYA-argF</i>) U169 <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>hsdR</i> 17 (<i>r_k</i> ⁻ , <i>m_k</i> ⁺) <i>phoA</i> <i>supE</i> 44 λ^- <i>thi</i> -1 <i>gyrA</i> 96 <i>relA</i> 1	
DH5 α F' (pRK2013)	DH5 α F' containing the helper plasmid pRK2013 with <i>tra</i> ⁺ , ColE1 replicon, Km ^r	Invitrogen, Karlsruhe, Germany Figurski and Helinski 1979
<i>Azoarcus</i> sp. strain		
BH72	Wild type	Reinhold et al. 1986
BHhexA2S	Δ <i>exaA2</i> with Ω cassette, Sm ^r	This work
BHhexA3K	<i>exaA3</i> ::Km cassette, Km ^r	This work
BHhexA2S-A3K	Δ <i>exaA2</i> with Ω cassette, <i>exaA3</i> ::Km cassette, Km ^r Sm ^r	This work
BHhexA1	<i>exaA1</i> ::pIMGG at position 937 bp, Km ^r	This work
BHhexA2	<i>exaA2</i> ::pIMGG at position 953 bp, Km ^r	This work
BHhexA3	<i>exaA3</i> ::pIMGG at position 1365 bp, Km ^r	This work
BHhexA4	<i>exaA4</i> ::pK18GGST at position 735 bp, Km ^r	This work
BHhexA5	<i>exaA5</i> ::pK18GGST at position 943 bp, Km ^r	This work
BHadhA	<i>adhA</i> ::pK18GGST at position 582 bp, Km ^r	This work
BHadhC	<i>adhC</i> ::pK18GGST at position 753 bp, Km ^r	This work
BHazo2700	<i>azo2700</i> ::pK18GGST at position 575 bp, Km ^r	This work

^a Sm^r and Km^r = resistant to spectinomycin and kanamycin, respectively.

tant role during the endophytic life of *Azoarcus* sp. strain BH72.

exaA2 and *exaA3* mutants are less competitive than the wild type during colonization of rice roots.

The expression of *exaA2* and *exaA3* inside of rice roots prompted the question of the importance of these two ADH for endophytic plant colonization by *Azoarcus* sp. strain BH72. To elucidate this question, marker exchange mutants of *exaA2* and *exaA3* were constructed (Table 1) and were used in single-strain set ups of rice infection tests. For both ADH mutants, no significant differences in endophytic colonization compared with the wild-type strain were observed (data not shown). Therefore, a competitive approach was used by inoculating rice roots with a mixture of equal amounts of BH72 and one mutant strain. This type of plant infection test revealed that both mutants (*BHxaA2S* and *BHxaA3K*) were significantly less com-

petitive than the wild type (Table 2). The results indicate the importance of *exaA2* and *exaA3* for plant colonization, conveying a competitive advantage over other colonizing bacteria lacking these genes. To address the question of whether the lack of both *adh* genes might affect colonization more strongly, a double mutant, *BHxaA2S-A3K*, was constructed (Table 1) and tested for endophytic establishment in rice roots in comparison with the wild type (Table 2). With only 14.5% of the colonizing bacteria being *BHxaA2S-A3K*, endophytic establishment was even less efficient than observed for single ADH mutants. This supported the assumption that ethanol is present in rice roots and an important carbon source for *Azoarcus* sp. strain BH72 during apoplastic colonization.

DISCUSSION

Although some of the details of the endophytic lifestyle of *Azoarcus* sp. strain BH72 are characterized (Krause et al. 2006), little is known about the physicochemical properties of root systems which allow BH72 to survive, multiply, and fix nitrogen. Particularly, the carbon sources which are utilized for endophytic activity are yet unidentified. In order to probe for nutrients provided by the plant in the vicinity of the bacteria, we applied an approach which makes use of inducible bacterial promoters. Therefore, an insertion library of *Azoarcus* sp. strain BH72 was constructed, whereby promoters were randomly fused to the marker gene *gfp*, similarly to what was described by Cowie and associates (2006). A microtiter-plate based screening for GFP activity was performed to identify *Azoarcus* genes whose expressions were induced in the presence of carbon sources, especially those thought to be available in the plant apoplast. When the culture medium was supplemented with ethanol, one recombinant *Azoarcus* strain showed high GFP fluorescence. Sequence analysis of the *gfp*-flanking region revealed that the trapped gene encodes a PQQ-dependent ADH, named ExaA3. Scanning of the complete *Azoarcus* genome discovered four additional genes encoding PQQ-dependent ADH, as well as three coding for NAD(P)-dependent ADH (Krause et al. 2006). Having eight potential ADH, the genus *Azoarcus* seems to be well equipped for different environmental conditions offering various alcohols.

Fig. 2. Functional analyses of the alcohol dehydrogenase (ADH) mutant strains in the free-living stage. Media used were supplemented with 30 mM malate or ethanol as carbon source, or no carbon source was added (w/o C source). **A**, Growth rate in culture of ADH mutants in an adapted SM (synthetic medium) under aerobic conditions or in a nitrogen-free SM medium with a microaerobic environment. Growth rates (average $\pm$ standard deviation [SD]) were calculated from at least four independent cultures when grown aerobically whereas, for microaerobically grown cultures, the growth rates were calculated from at least two independent experiments measured in triplicate. Asterisks show significant differences between mutants versus wild type ($P < 0.001$). **B**, Promoter activity of the ADH-encoding genes in nitrogen-free SM under aerobic or microaerobic conditions. β -Glucuronidase activity is given in Miller units (average $\pm$ SD) determined from at least four independent cultures measured in duplicate. Different letters above bars indicate significance among the average ($P < 0.001$).

Fig. 3. Malate repression of ethanol-induced alcohol dehydrogenase (ADH) expression. An *Azoarcus* sp. was cultivated under aerobic conditions in nitrogen-free SM (synthetic medium) supplemented with 30 mM malate, 30 mM ethanol, or a mixture of 3 mM malate and 30 mM ethanol. β -Glucuronidase activity is specified as Miller units (average $\pm$ standard deviation) determined from at least four independent cultures measured in duplicate. Significantly different activities are indicated by different letters above the bars ($P < 0.001$).

Quite a number of bacteria can obtain their energy for growth by oxidizing ethanol. Therefore, the use of ethanol as carbon source is not exceptional to *Azoarcus* strains. Also, among the nitrogen-fixing bacteria, this is not a surprising feature; for example, some rhizobia and bradyrhizobia are capable of growing on this carbon source (Sadowsky and Bohlool 1986). Additionally, the presence of multiple ADH is not unique to *Azoarcus* sp. strain BH72 (for example, *Pseudomonas butanovora*) which, in alcohol-utilizing bacteria, probably reflects the habitat to which the organism is adapted (Radianingtyas and Wright 2003; Vanganai and Arp 2001). The habitat of *Azoarcus* sp. is mainly the apoplastic space of the aerenchyma of grass roots grown under waterlogged conditions (Reinhold-Hurek and Hurek 1998). Rice, one of the grasses which can be colonized by *Azoarcus* sp. strain BH72, grown under these conditions, is subjected to hypoxia and even to anoxia, which induces morphological modification and metabolic changes. Adaptation of the metabolism includes a switch of the respiration from the oxidative to the fermentation pathway, leading to production and accumulation of ethanol (Agarwal and Grover 2006; Mustroph and Albrecht 2003). However, in aerated rice roots, ethanol accumulation can also be observed (Mustroph et al. 2006). Because ethanol can pass directly through cell membranes by simple diffusion, this compound would appear in the plant apoplast and, therefore, would be available for *Azoarcus* strains.

Under pure culture conditions, expression of three of the eight ADH from *Azoarcus* sp. strain BH72 was induced by ethanol, of which two, the PQQ-dependent ADH ExaA2 and ExaA3, showed even higher induction under nitrogen-fixing conditions. Induction of gene expression by ethanol indicates that these ADH are involved in the oxidation of ethanol, feeding it into the central metabolism pathway as carbon and an energy source for bacterial growth. Substrate specificity of the other five ADH remains unclear because, in culture, no induction of gene expression could be observed by either ethanol or methanol, propanol, or butanol. Further studies will attempt to clarify the substrate specificity and the physiological function of these additional ADH in *Azoarcus* sp. strain BH72.

Strong expression of the two PQQ-dependent ADH (ExaA2 and ExaA3) was also visible inside rice roots. Additionally, the presence of these ADH conferred an advantage in endophytic

plant colonization under competitive conditions. Therefore, ethanol seems to occur in sufficiently high concentration within the plant to direct bacterial ADH expression. Interestingly, in pure culture, induction of ADH by ethanol was subjected to catabolite repression by the presence of fumarate, α -ketoglutarate, lactate, malate, or pyruvate. The involvement of catabolite repression in *adh* gene expression was already demonstrated for *Corynebacterium glutamicum* (Kotrbova-Kozak et al. 2007) and, therefore, is not an unique feature for *Azoarcus* sp. strain BH72. However, while these carbon sources can be metabolized by strain BH72 (Reinhold-Hurek et al. 1993b) and while expression of ExaA2 and ExaA3 occurs within the plant, fumarate, α -ketoglutarate, lactate, malate, or pyruvate are either absent or at least present only in very low concentrations in the apoplastic fluid under the conditions studied. Thus, it is likely that ethanol serves as abundant carbon source for the endophytic life of strain BH72.

Moreover, additional regulatory circuits of relevance in the plant-microbe interaction might be affected by the presence of ethanol. The activity of hydrolytic enzymes that are likely to degrade plant cell wall components such as surface-bound exoglycanase and endoglucanase is enhanced in strain BH72 in the presence of ethanol (Reinhold-Hurek et al. 1993a). Indeed, the expression of the gene encoding the endoglucanase EglA was induced by ethanol. Because this endoglucanase was found to be involved in intracellular colonization of and systemic spreading in rice (Reinhold-Hurek et al. 2006), the endophytic interaction is likely to be affected by ethanol beyond catabolism.

In rice roots, 98% of the fermentatively produced ethanol is immediately released from the tissue into the environment (Bertani et al. 1980; Mustroph et al. 2006). This may serve for *Azoarcus* strains as an attractant to enter the roots. Once inside the root, the endophytes consume the ethanol and, thus, might help the plant to keep concentrations of ethanol below the critical level of toxicity. The plant benefit of this consumption might be an increased tolerance to hypoxic conditions. Experimental analyses need to be performed to elucidate this potentially additional role of *Azoarcus* sp. strain BH72 in the endophytic interaction with rice.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacterial strains used are listed in Table 1. *Escherichia coli* was grown in Luria-Bertani medium (Sambrook et al. 1989) at 37°C with appropriate antibiotics added. *Azoarcus* sp. strain BH72 and its mutant strains were cultivated aerobically at 37°C in a modified VM (Voll medium), in which the beef extract was replaced by 0.3% peptone containing malate (Reinhold-Hurek et al. 1993b) or ethanol (Hurek et al. 1995; Reinhold-Hurek et al. 1993a) as carbon source. Additionally, growth of BH72 was performed in an adapted SM (synthetic medium) (Reinhold et al. 1985) devoid of yeast extract and containing ethanol (6 ml/liter) instead of malate. Antibiotics used were kanamycin and spectinomycin at a final concentration of 30 and 40 µg/ml, respectively. To determine the GUS or GFP activity, bacterial cells were culti-

Fig. 4. Identification of ExaA2 and ExaA3 during interaction of *Azoarcus* sp. strain BH72 with rice or an ascomycete. Extracts of total proteins from **A**, uninoculated control rice roots; **B**, rice roots inoculated with BH72; **C**, Strain BH72 grown under microaerobic nitrogen-fixing conditions; or **D**, Strain BH72 co-cultivated with the ascomycete *Acremonium alternatum* strain 2003 were separated by two-dimensional gel electrophoresis. Shown are close-ups of stained gels. Circled protein spots are missing in the corresponding control. Identification was performed by matrix-assisted laser desorption ionization time-of-flight or liquid chromatography tandem mass spectrometry.

Table 2. Bacterial competition during plant colonization

Strain in competition with BH72	Percentages of colonizing bacteria (average $\pm$ SD) ^a	P value
BHExaA2S	30.3 $\pm$ 4.6	4.9 $\times$ 10 ⁻⁴
BHExaA3K	32.7 $\pm$ 11.7	1.1 $\times$ 10 ⁻²
BHExaA2S-A3K	14.5 $\pm$ 4.4	3.9 $\times$ 10 ⁻⁵

^a Analyses based on a minimum of 27 plants per mixture combined from three independent trials. SD = standard deviation.

vated aerobically in nitrogen-free SM with different carbon sources at a final concentration of 30 mM. Microaerobic growth was performed under nitrogen-fixing conditions in nitrogen-free SM with ethanol in either a rubber-stopper-sealed Erlenmeyer flask with an initial concentration of 1.2% O₂ in the N₂ headspace or a 2-liter fermenter (Biostat B; Braun Biotech, Melsungen, Germany) with a regulated oxygen supply of 0.12% in N₂. Co-cultivation of strain BH72 with the ascomycete *Acremonium alternatum* 2003 was carried out as described by Hurek and associates (1995) at 30°C until oxygen in the gas phase had diminished to 4%.

DNA protocols and sequence data analysis.

Recombinant DNA techniques were performed following established protocols (Sambrook et al. 1989). DNA sequencing was carried out by the dideoxynucleotide chain method on an ALFexpress automated sequencer (GE Healthcare, Freiburg, Germany).

Sequence comparisons were conducted through the BLAST program (Altschul et al. 1997) of the National Center for Biotechnology Information (NCBI; Bethesda, MD, U.S.A.). Domain structures of proteins were analyzed by the conserved domain database at NCBI (Marchler-Bauer et al. 2007) or by the Pfam database of the Wellcome Trust Sanger Institute (Hinxton, U.K.) (Finn et al. 2006). Signal peptides were predicted with SignalP 3.0 (Bendtsen et al. 2004) while transmembrane helices were identified by using TMHMM 2.0 (Krogh et al. 2001), both from the Center for Biological Sequences Analysis (Lyngby, Denmark).

Construction of a transcriptional fusion library to *gfp* and *gusA* in *Azoarcus* sp. strain BH72.

A plasmid library was constructed in pIMGG (Fig. 1) by digestion of the vector with *SalI* and partial fill ups of the overhanging ends with dCTP and dTTP. Chromosomal DNA of *Azoarcus* sp. strain BH72 was partly digested with *Sau3AI* and size fractionated through gel electrophoresis to 2 to 5 kb. Overhangs were filled in by using dATP and dGTP. After ligation, recombinant plasmids were electroporated into *E. coli* DH10B generating 1.5×10^5 colonies. Each plasmid was separately mobilized into strain BH72 via triparental conjugation. In all, 1.25×10^3 recombinants were obtained by selecting for the vector-encoded Km^r marker, indicating a single-crossover event into the *Azoarcus* genome.

Microtiter-plate based screening for GFP expression of the library.

Recombinant *Azoarcus* clones were transferred into DeepWell plates (with 96 wells each) containing 1 ml of VM and were grown overnight at 37°C. After two times of washing the bacteria with nitrogen-free SM without carbon source, cells were subjected in parallel to flat-bottom microtiter plates with 100 µl of nitrogen-free SM containing 52 different carbon sources at a final concentration of 30 mM followed by an incubation of 4 h at 37°C. Expression of GFP was directly monitored by scanning the microtiter-plates with the fluorescence imager Typhoon 8600 (GE Healthcare, Freiburg, Germany) (excitation wavelength 532 nm, emission filter 526 SP). Relative fluorescence was normalized against an optical density at

Fig. 5. Expression profile of the bacterial genes *exaA2* or *exaA3* in rice roots. The figure shows representative photographs. Fluorescence detection inside of **A** and **D**, inoculated rice roots or **B** and **E**, cultures supplemented with ethanol. **C** and **F**, No fluorescence was visible when malate was used as carbon source. Roots of rice seedlings were inoculated with BH*exaA2S* and grown for 14 days gnotobiotically whereas, for BH*exaA3K*, seedlings were placed into bacterial suspension and incubated for 3 days in the growth chamber (short-term invasion analysis). Bacterial cultures were grown overnight. **D** is taken from Reinhold-Hurek and associates (2007). Settings of the fluorescence microscope and the video camera were equal for micrographs of roots and bacterial cultures. Scale bars = 50 µm.

570 nm (OD₅₇₀) obtained with the Multiskan Ascent (Thermo Scientific, Waltham, MA, U.S.A.).

Disruption of genes encoding ADH.

exaA2 and *exaA3* were disrupted by marker exchange mutagenesis. DNA segments to be deleted were replaced by an antibiotic resistance cassette (Sm^r/Sp^r for *exaA2* obtained from the plasmid pH45Ω [Prentki and Krisch 1984] and Km^r for *exaA3* taken from pUC4K [GE Healthcare]). The recombinant plasmids were either electroporated into the *Azoarcus* sp. (*exaA3* on pUC19) (Yanisch-Perron et al. 1985) or used for subcloning of the modified DNA fragment into the mobilizable vector pK18mob2 (Kirchner and Tauch 2003), which was then transferred into *Azoarcus* sp. by triparental conjugation (*exaA2*). Double-crossover events at homologous sites resulted in antibiotic-resistant strains (Table 1). The plasmid integration mutagenesis was performed to generate transcriptional fusions of the genes of interest to *gfp* and *gusA* by amplifying appropriate parts of the genes followed by cloning of the polymerase chain reaction products into the vector pIMGG or pK18GGST (Fig. 1). The resulting plasmids were transferred into *Azoarcus* sp. by triparental conjugation. The vector-encoded Km^r marker allowed selection of strains with the plasmid integrated into the chromosome (Table 1). Correct integration events were confirmed by Southern blot analysis.

Analyses of growth rates of mutant strains.

To evaluate growth of the mutants compared with the wild type, strains were precultured in VM with ethanol or malate overnight at 30 or 37°C. Cells were centrifuged, washed twice, and resuspended in adapted SM for aerobic growth or in nitrogen-free SM for microaerobic growth in the fermenter. Media were supplemented with carbon sources to a final concentration of 30 mM. Cultures were adjusted to an OD₆₀₀ of 0.05/0.01 (aerobic/microaerobic) and further incubated at 37°C. Culture samples were taken at different time points during exponential growth, and OD was determined at 600 nm. Growth rates were calculated for each culture and averaged. Data were statistically analyzed by a two-tailed *t* test.

GUS activity in free-living *Azoarcus* mutant strains.

Cultures were grown in VM with malate at 30°C overnight. Cells were collected by centrifugation, washed twice with nitrogen-free SM without carbon source, and finally adjusted to an OD₆₀₀ of 0.3 in nitrogen-free SM supplemented with 30 mM different carbon sources. After additional incubation of 6 h under aerobic or nitrogen-fixing microaerobic conditions at 37°C, two 1-ml samples were taken. Bacterial cells were pelleted and resuspended in 2 ml of GUS buffer (50 mM sodium phosphate, pH 7, and 10 mM β-mercaptoethanol). To permeabilize the bacterial cells, 0.5 ml of the suspension was treated for 10 min at 37°C with 50 μl of lysis buffer (0.5× TE, pH 8, and 0.05% sodium dodecyl sulfate) while the OD₆₀₀ of the cells was determined from the remaining suspension. Quantitative GUS activity assays were performed with *p*-nitrophenyl-β-D-glucuronide as described by Jefferson and associates (1986). Activity of the GUS was calculated as Miller units from each culture. The average was calculated and significance was determined by using the Tukey-Kramer multiple comparison test.

Plant growth condition and inoculation tests.

Rice plants used were *Oryza sativa* subsp. *indica* 'IR36' obtained from the International Rice Research Institute (Los Banos, Philippines). Rice grains were surface sterilized and germinated on agar plates according to Hurek and associates (1994) with the modifications that the washing steps with distilled water after sterilization were elongated to 1 h and per-

formed three times while the germination plates contained only 10% agar. Germination took place for 3 to 4 days in the dark at 37°C succeeded by an additional day in the growth chamber with a day time of 14 h at 30°C and 80% relative humidity, a light intensity of 23 kLux, and a night time of 10 h at 30°C. Germinated seedlings were inoculated with individual bacterial strains for the single strain set-up or for the competitive approach with a 1:1 mixture of the wild-type and a mutant strain, as described by Egner and associates (1999), with the exception that the nitrogen-free plant medium contained malate at 20 mg/liter. Cultivation of plants was performed gnotobiotically for 14 days in the growth chamber or in the greenhouse where conditions were similar to the growth chamber other than a 12-h light time in addition to the natural day-night rhythm. For short-term invasion analyses, seedlings were placed into 1 ml of bacterial suspension with 5 × 10⁸ cells/ml and incubated for 3 days in the growth chamber (Reinhold-Hurek et al. 2006). Expression of GFP was monitored by fluorescence microscopy with a Zeiss Axioplan 2 (Carl Zeiss AG, Jena, Germany) according to Egner and associates (1998). To quantify endophytic colonization of rice roots, plants were harvested after 14 days. Roots were treated by ultrasonification and homogenized (Böhm et al. 2007). Dilutions of the homogenate were plated out on SM to determine CFU counts as degree of colonization. In the competitive approach, 50 colonies were randomly transferred in parallel to VM-ethanol plates with and without appropriate antibiotics in order to distinguish the mutant strain from the wild type. All competition experiments were repeated in three independent trials. The percentage of colonizing mutant strain was calculated, averaged, and statistically analyzed by a two-tailed *t* test.

Protein analyses and identification.

Preparation of total protein extracts from control and treated roots as well as separation of the proteins by two-dimensional gel electrophoresis and protein identification through matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) or liquid chromatography tandem mass spectrometry were performed as described by Miché and associates (2006).

For extraction of bacterial proteins, *Azoarcus* sp. strain BH72 grown in co-culture with the ascomycete was first separated from the fungal mycelium and then pelleted (Hurek et al. 1995). Cells were resuspended in 50 mM sodium phosphate buffer (pH 7) and disrupted by four passages through a French press (1,500 lb/in.²). After centrifugation (20 min at 20,000 × *g*, 4°C), the extracts were fractionated by ultracentrifugation (2 h at 200,000 × *g*, 4°C). Proteins from the supernatant were precipitated by trichloroacetic acid. Pellets were dissolved in extraction buffer and further processed as described by Hauberg and associates (2010). Then, proteins were subjected to two-dimensional gel electrophoresis (Hauberg et al. 2010; Hurek et al. 1995) and further identified by MALDI-TOF with the Proteome-Analyzer 4700 (Applied Biosystems, Foster City, CA, U.S.A.) (Hauberg et al. 2010).

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