The Regulatory Network Controlling Ethanol-Induced Expression of Alcohol Dehydrogenase in the Endophyte *Azoarcus* sp. Strain BH72

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The habitat of the nitrogen-fixing endophyte *Azoarcus* sp. strain BH72 is grass roots grown under waterlogged conditions that produce, under these conditions, ethanol. Strain BH72 is well equipped to metabolize ethanol, with eight alcohol dehydrogenases (ADHs), of which ExaA2 and ExaA3 are the most relevant ones. *exaA2* and *exaA3* cluster and are surrounded by genes encoding two-component regulatory systems (TCSs) termed ExaS-ExaR and ElmS-GacA. Functional genomic analyses revealed that i) expression of the corresponding genes was induced by ethanol, ii) the genes were also expressed in the rhizoplane or even inside of rice roots, iii) both TCSs were indispensable for growth on ethanol, and iv) they were important for competitiveness during rice root colonization. Both TCSs form a hierarchically organized ethanol-responsive signal transduction cascade with ExaS-ExaR as the highest level, essential for effective expression of the ethanol oxidation system based on ExaA2. Transcript and expression levels of *exaA3* increased in *tcs* deletion mutants, suggesting no direct influence of both TCSs on its ethanol-induced expression. In conclusion, this underscores the importance of ethanol for the endophytic lifestyle of *Azoarcus* sp. strain BH72 and indicates a tight regulation of the ethanol oxidation system during root colonization.

Endophytic bacteria are known to colonize the plant interior, often in high numbers, without inducing strong plant defense responses (Reinhold-Hurek and Hurek 2011). One of the best analyzed endophytes is the diazotrophic betaproteobacterium *Azoarcus* sp. strain BH72, originally isolated from Kallar grass (*Leptochloa fusca* L. Kunth) (Reinhold et al. 1986). The habitat of strain BH72 is the root apoplast of grasses, including rice, grown under waterlogged conditions in which the physico-chemical properties of the microenvironment are suitable for proliferation and nitrogen fixation. Although the genome sequence of strain BH72 is available (Krause et al. 2006), the molecular mechanisms relevant for an efficient beneficial interaction with its host plant are not yet well understood. Bacterial features so far known to be essential for the endophytic lifestyle are i) a type IV

pilus-dependent adhesion to the root surface (Dörr et al. 1998), ii) a PilT- and PilX-conveyed twitching motility (Böhm et al. 2007; Shidore et al. 2012), iii) an endoglucanase EglA-facilitated entry into the root epidermis (Reinhold-Hurek et al. 2006), iv) a flagella-mediated endophytic colonization (Buschart et al. 2012), v) a type VI secretion system-regulated colonization capacity (Shidore et al. 2012), vi) an autotransporter-contributed establishment inside roots (Sarkar et al. 2017), and vii) a metabolic adaptation to the plant environment (Sarkar and Reinhold-Hurek 2014; Sarkar et al. 2017; Shidore et al. 2012).

One of the metabolic adaptations important for the rice-endophyte interaction is the adjustment to the presence of ethanol. Rice grown in paddy fields is subjected to hypoxia and even to anoxia, which leads to fermentation provoking production and accumulation of ethanol (Agarwal and Grover 2006; Mustroph and Albrecht 2003). *Azoarcus* sp. strain BH72 has a strictly respiratory type of metabolism but is, nevertheless, able to grow aerobically and microaerobically on ethanol as the sole source of carbon and energy (Krause et al. 2006, 2011). The genome of strain BH72 comprises eight genes encoding alcohol dehydrogenases (ADHs). Functional genomics using plasmid integration mutagenesis coupled with expression profiling, based on the plasmid-borne *gfp* (green fluorescent protein) and *gusA* marker genes, revealed an ethanol-induced expression of three ADHs, of which two were also strongly expressed inside rice roots. Disruption of these two genes, encoding pyrrolo-quinoline quinone (PQQ)-dependent ADHs named ExaA2 and ExaA3, diminished competitiveness during endophytic colonization of rice roots. Therefore, these two ADHs are important for the endophytic lifestyle of strain BH72 (Krause et al. 2011).

For adequate adaptation to metabolic requirements in the habitat, endophytes have to react quickly by precise regulation of gene expression in response to various environmental stimuli. In order to achieve this assignment, bacteria use a variety of signal transduction systems, including two-component systems (TCSs). Components of the TCSs are histidine sensor kinases, which undergo autocatalytic phosphorylation upon an environmental stimulus, and cognate response regulators, to which the phosphoryl group is transferred, resulting in transcriptional regulation of target genes. According to P2CS, a TCS database, the genome of *Azoarcus* sp. strain BH72 encodes 63 histidine sensor kinases and 71 response regulators (Ortet et al. 2015). Thus, strain BH72 is well-equipped to form sophisticated signal transduction networks reacting to a wide range of environmental signals.

Until now, little is known about these signal transduction networks in *Azoarcus* sp. strain BH72. Here, we describe, based on functional genomics, the first steps of a hierarchical network

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controlling the metabolism of ethanol, an important carbon source in the rice-endophyte interaction.

RESULTS

Chromosomal arrangement of genes involved in ethanol oxidation.

The genome of *Azoarcus* sp. strain BH72 codes for eight different ADHs, two of which—the PQQ-dependent ADHs ExaA2 and ExaA3—were shown to be of primary importance for ethanol oxidation and endophytic life with rice (Krause et al. 2011). Interestingly, *exaA2* and *exaA3* are situated in the same genetic locus, surrounded by genes coding for two TCSs, named ExaS-ExaR and Elms-GacA (Fig. 1). The reading frame of *exaS* overlaps the one of *exaR* by 3 bp, indicating that these two genes are forming an operon. In contrast, *elms* is separated from *gacA* by 213 bp. A computer-based evaluation of this intergenic region predicted a σ^{70} promoter sequence upstream of *gacA*. Indeed, reverse transcription-polymerase chain reaction (RT-PCR) analysis bridging the two respective genes confirmed that they are not cotranscribed (data not shown). Apart from *Azoarcus olearius* DQS-4^T (Chen et al. 2013; Faoro et al. 2017), the closest relative of *Azoarcus* sp. strain BH72, none of the so-far-sequenced bacteria has a similar arrangement of genes involved in ethanol oxidation.

The response regulators (ExaR, GacA) carry a helix-turn-helix DNA-binding domain of the LuxR type, indicating that they serve as transcriptional activators. They have an overall sequence identity to each other of 45%. ExaS and Elms are classical histidine sensor kinases with a HAMP domain, a domain known to be part of integral sensor kinases and to be involved in transmission of periplasmic signals to the cytoplasm (Aravind and Ponting 1999). In fact, the prediction of two transmembrane helices in ExaS and Elms indicates a membrane-bound localization with a periplasmic loop. Their

overall sequence identity is only 28%. Both TCSs were found to have significant sequence identity of up to 44% to EraR-EraS of *Pseudomonas aeruginosa*, a TCS regulating expression of the PQQ-dependent ADH ExaA (Schobert and Görisch 2001). Taking the genomic location and the sequence identity in consideration, a potential role of ExaS-ExaR and Elms-GacA in regulation of the ADH expression in *Azoarcus* sp. strain BH72 could be postulated.

Growth of putative regulatory mutants on ethanol.

In order to clarify the role of the two TCSs in the ethanol metabolism, mutants in each TCS were constructed. According to the predicted operon structure mutants were separately generated in *gacA* and *elms* through in-frame deletion, while *exaRS* were inactivated together by a combination of deletion and insertion of the streptomycin- and spectinomycin-resistant (*Sm^r/Sp^r*) antibiotic resistance cassette (Fig. 1A). Growth behavior of the mutant strains was determined on malate- and ethanol-containing media, both being major carbon sources for *Azoarcus* sp. strain BH72 (Reinhold-Hurek et al. 1993). Inactivation of these genes did not affect cultivation on malate but prevented the mutants growth on ethanol (Fig. 2A). As mutant BH*exaRS*s might show a possible polar effect on *azo2969*—the gene directly downstream of *exaR*—the plasmid integration mutant BH*azo2969tg* was designed (Fig. 1A). Growth of this mutant strain on ethanol-containing media was with a growth rate of 0.40 ± 0.04 , not significantly reduced in comparison with the wild type. Therefore, the lack of growth on ethanol could clearly be assigned to the absence of the TCS ExaS-ExaR.

Interestingly, the observed growth phenotype of all TCSs mutant strains was more severe than the one obtained by disruption of *exaA2* or *exaA3*, which still showed 19 or 57% of the wild type growth on ethanol (Krause et al. 2011), and even more than the one determined for a *exaA2-exaA3* double mutant

Fig. 1. The *exa2-exa3* gene cluster. Structural genes encoding pyrrolo-quinoline quinone-dependent alcohol dehydrogenases are shown in black while genes coding for two-component regulatory systems are marked in gray. 1 cm = 1,000 bp. **A**, Mutants constructed in this study. Positions of transcriptional fusions to *gfp-gusA*, *tdTomato-gusA*, or *tdTomato* are labeled by triangles and are indicated by the corresponding names. Areas deleted in in-frame deletion mutants or replaced by the streptomycin- and spectinomycin-resistant antibiotic resistance cassette in marker exchange mutants are displayed. All mutant names are given. **B**, The ethanol-responsive signal transduction pathway forming a hypothetical hierarchical network controlling *exaA2* expression. Response regulators are illustrated by circles. Hexagons display sensor kinases.

with a growth rate of 0.05 ± 0.013 . Therefore, in *Azoarcus* sp. strain BH72 both TCSs are essential for the ethanol utilization as carbon source.

Expression profile of the sensor kinases ExaS and ElmS and the response regulator GacA in free-living cells.

Transcriptional fusions of the TCSs encoding operons with the reporter gene *gusA* were constructed to analyze their expression levels under various conditions (Fig. 1A). In *Azoarcus* sp. strain BH72, the expression of *exaS*, *elmS*, and *gacA* was significantly enhanced by the addition of ethanol, as carbon source, to the growth medium versus malate (Fig. 2B), indicating that these genes are members of the ethanol-induced regulon. This ethanol-regulated induction was independent of aerobic or nitrogen-fixing microaerobic growth conditions and varied between factors of three to ten based on the gene and the condition analyzed. While the overall expression of *elmS* and *gacA* did not differ between aerobic and nitrogen-fixing microaerobic conditions, *exaS* showed a significantly higher expression in the nitrogen-fixing microaerobic environment compared with the aerobic one (defined by the *t* test, $P < 0.001$). This twofold increased expression was also detectable in N-replete, microaerobic, ethanol-containing growth media with $1,805 \pm 254$ Miller units, suggesting that *exaS* not only belongs to the ethanol-induced regulon but, also, overlaps with the oxygen-responsive regulon.

ExaRS, GacA, and ElmS are involved in induction of expression by ethanol of *exaA2* but not of *exaA3*.

The ethanol-induced expression of *exaS*, *elmS*, and *gacA* as well as the sequence identity to EraR-EraS of *P. aeruginosa* (Schobert and Görisch 2001) evoke the hypothesis that these two TCSs of strain BH72 are also involved in the ethanol-responsive signal cascade. Endpoints of this signal cascade are presumably the ADH-encoding genes *exaA2* and *exaA3*, which showed drastically increased expression by the addition of ethanol to the culture media in comparison with malate-containing media (Krause et al. 2011). Therefore, the ethanol-induced expression of *exaA2* and *exaA3* was studied in the TCS deletion mutant strains (Fig. 1A). Using chromosomally integrated transcriptional fusions of both genes to *gusA* as well as RT-PCR analyses revealed an alteration in gene expression in the presence of ethanol (Fig. 3A and B). Expression of *exaA2* was lower or noninduced in all generated putative regulatory mutants, as compared with the wild type. Interestingly, neither

was β -glucuronidase activity detectable nor were transcripts of *exaA2* visible in the mutant, where the TCS ExaR-ExaS was inactivated. Deletion of *elmS* or *gacA* lowered *exaA2*-driven β -glucuronidase activity to 20 and 30%, respectively, of the wild-type level. This reduced expression in the presence of ethanol was also seen for the *exaA2* transcript levels in the respective mutant strains. Thus, both TCSs are required for effective, ethanol-induced expression of *exaA2*.

On the contrary, the ethanol-induced *exaA3* expression, as determined by β -glucuronidase activity, was significantly elevated by a factor of 1.8 in the *exaRS* and *elmS* deletion mutants, in comparison with the wild type. Equally, *exaA3* transcript levels increased drastically. When the response regulator GacA was inactivated, no significant changes in *exaA3* promoter activity nor in *exaA3* transcript levels could be observed (Fig. 3A and B). This observed elevated induction of *exaA3* expression by ethanol in the mutant strains raised the question whether the absence of ExaA2 was compensated for by a more severe induction of *exaA3* expression through ethanol. In order to verify this assumption, the *exaA3-gusA* transcriptional fusion was integrated into the genome of BHexaA2s, a mutant strain where part of *exaA2* was replaced by a Sm^r/Sp^r antibiotic resistance cassette (Krause et al. 2011). As *exaA3* expression in this mutant strain was with $3,782 \pm 775$ Miller units identical to the one in the wild type, it can be stated that the increased expression of *exaA3* is not directly linked to an absence of ExaA2. The inability of ethanol consumption in the *tcs* deletion mutants seems to be adjusted at another step in the metabolism and is then transmitted from there by one or more unknown regulators towards *exaA3*.

ExaRS, ElmS, and GacA forming a hierarchical ethanol-responsive signal cascade.

Reporter gene fusions of *exaS*, *elmS*, or *gacA* integrated into the genomes of the different TCS deletion mutant strains allowed the determination of the mutual interference with each other on their expression. The results showed that *exaA3* expression does not depend on the presence of the TCS ElmS-GacA (Fig. 3C). Also, expression of *gacA* appears to be independent of the TCS ExaS-ExaR as well as the sensor kinase ElmS. On the contrary, the sensor kinase ExaS and the response regulator ExaR are required for controlling the ethanol-induced expression of *elmS*. Therefore, the TCS ExaS-ExaR seems to represent a higher level of regulatory components in the ethanol-responsive signal cascade than the TCS ElmS-GacA (Fig. 1B).

Fig. 2. Analyses of strains carrying mutations in genes encoding two-component regulatory systems. Strains were cultured in synthetic medium (SM) under aerobic conditions or in a nitrogen-free SM with a microaerobic environment, both supplemented with ethanol (black columns) or malate (white columns). **A**, Growth of free-living *Azoarcus* strains carrying deletion of the desired gene. Growth rates (average $\pm$ standard deviation) were determined from at least four independent cultures. Asterisks show significant differences between mutants versus wild type ($P < 0.001$). **B**, Promoter activity of the desired genes transcriptionally fused to *gusA* during the free-living stage. Activity is given as β -glucuronidase activity in Miller units (average $\pm$ standard deviation) determined from five independent cultures measured in duplicate. Asterisks indicate significantly higher β -glucuronidase activity in ethanol-containing medium compared with malate medium ($P < 0.001$).

A gene, *tbd2978*, located upstream of *elmS* (Fig. 1A), is cotranscribed with it. Expression analyses of this gene revealed that the ethanol-induced expression is not altered in the *elmS* deletion mutant compared with the one observed in the wild type

(data not shown). Apparently, the sensor kinase ElmS is not essential for its own expression.

Analysis of importance of ExaS-ExaR and ElmS-GacA for rice root colonization.

ExaA2 conveyed a competitive advantage to *Azoarcus* sp. strain BH72 during endophytic colonization (Krause et al. 2011). The participation of the TCSs ExaS-ExaR and ElmS-GacA in controlling the *exaA2* expression raised the question of the significance of these systems for rice root colonization. To study this question, marker exchange mutants of *elmS* and *gacA* using the *Sm^r/Sp^r* antibiotic resistance cassette were constructed, based on the in-frame deletion mutants (Fig. 1A). For *exaS*, the mutant described above was used. In a competitive approach, roots of rice seedlings were inoculated by a mixture of equal amounts of *Azoarcus* sp. strain BH72 and one mutant strain. The result disclosed that the wild type significantly colonized the roots, by a factor of 2.2, better than any of the mutant strains (Table 1). This factor was identical to the one achieved when the ADH-encoding genes *exaA2* or *exaA3* were deleted (Krause et al. 2011). Therefore, these two TCSs are as important for effective root colonization as the ADHs themselves, most probably by also regulating in planta the gene expression of *exaA2*.

This assumption implies that the two TCSs are expressed in planta. In order to verify the expression, transcriptional fusions of *exaS*, *elmS*, and *gacA* to *tdTomato* were constructed, identical to the previously described strategy of *gfp-gusA* fusions (Fig. 1A). *TdTomato* was chosen as a fluorescent marker on the basis of its exceptional brightness, as expression of the *tcs* in free-living strain BH72 was insufficiently high (Fig. 2B) for GFP-based single-cell microscopic visibility (data not shown). As seen by the red fluorescence signal, all three bacterial genes were expressed during rice root colonization (Fig. 4). Expression of *exaS* was detectable at the rhizoplane and at the first layer of the endorhizosphere in which the expression of *elmS* and *gacA* was also observable. Therefore, the different microhabitats of a plant-microbe interaction, the root interior as well as the interface between the root and the rhizosphere, provide suitable conditions for the expression of the two *tcs* systems, which then might lead in planta to the activation of *exaA2* expression.

DISCUSSION

Azoarcus sp. strain BH72 is metabolically not very versatile. As an adaption to the plant-compatible endophytic lifestyle, energy can be mainly obtained by oxidation of dicarboxylic acids or ethanol (Reinhold-Hurek et al. 1993). Having eight ADHs, of which the PQQ-dependent ADHs ExaA2 and ExaA3 are the most important ones for growth on ethanol, strain BH72 seems to be well-prepared for ethanol metabolism (Krause et al. 2011).

The close genomic vicinity of *exaA2* and *exaA3* is not unique to *Azoarcus* sp. strain BH72. This was also found in different *Pseudomonas* strains (Tribelli et al. 2015), which are able to grow on ethanol as carbon source as well. In strain BH72, adjacent to the *exaA2-exaA3* cluster, are four genes coding for

Fig. 3. Influence of the two-component regulatory systems on the expression of genes important for ethanol oxidation. For determination of promoter activities, the β -glucuronidase activity test was used after 6 h of growth under aerobic conditions in synthetic medium containing ethanol. Activity is specified as Miller units (average $\pm$ standard deviation) calculated from five independent cultures measured in duplicate. Significantly different activities in comparison with the activity in the wild type are indicated by asterisks ($P < 0.001$). For determination of transcript levels semiquantitative reverse transcription-polymerase chain reaction analyses were performed on total RNA extracted from aerobically grown overnight cultures in Voll medium containing ethanol. **A**, Promoter activity of the alcohol dehydrogenase-encoding genes *exaA2* and *exaA3* in wild type and different two-component regulatory system (TCS) mutant strains. **B**, Transcript levels of *exaA2* and *exaA3* in wild type and different TCS mutant strains. Representative results of cycle 26 or 28 are shown. Corresponding controls without reverse transcriptase showed no presence of DNA in the total RNA samples (data not shown). **C**, Expression profile of *exaS*, *elmS*, and *gacA* in different TCS mutant strains.

Table 1. Bacterial competition during endophytic root colonization

Strain in competition with BH72	Colonizing bacteria (in %) ^a	<i>P</i> value
BH <i>exaRSs</i>	30.7 $\pm$ 5.4	9.4 $\times$ 10 ⁻⁴
BH <i>elmSs</i>	31.5 $\pm$ 2.5	5.7 $\times$ 10 ⁻⁵
BH <i>gacAs</i>	31.3 $\pm$ 1.2	3.2 $\times$ 10 ⁻⁶

^a Analyses based on three independent experiments with a total of 33 plants per mixture. Shown is the average $\pm$ standard deviation.

two localized TCSs. Both TCSs were shown to be essential for the ethanol-responsive signal transduction pathway regulating effective expression of *exaA2*. Surprisingly, none of the TCSs were involved in regulation of ethanol-induced expression of *exaA3*, although these two ADHs have the same substrate specificity (Krause et al. 2011).

The organization of the TCSs in this signal transduction pathway is hierarchical (Fig. 1B), encompassing, until now, two levels, similar to the regulatory networks of the ethanol oxidation system of *P. aeruginosa* (Gliese et al. 2004; Mern et al. 2010) and *P. putida* (Promden et al. 2009) as well as of the methanol oxidation system of *Methylobacterium extorquens* (Springer et al. 1997). The highest level in this hypothetical signal cascade is the TCS ExaS-ExaR, which is important for the ethanol-induced expression of *elmS*. Then ElmS together with GacA, whose ethanol-induced expression is independent of ExaS-ExaR, directly or indirectly regulates the *exaA2* expression in response to the availability of ethanol.

The proposed hierarchical network is based on the assumption that the sensor kinases ExaS and ElmS form TCSs with their cognate response regulators ExaR and GacA, respectively, without functional interchange between each other. This non-functional interchange is experimentally supported by the inability of the mutant strains to grow on ethanol. On the other hand, the proposed hierarchical network fails to explain the observed inability of all regulatory mutants to grow on ethanol as sole carbon source. As the regulatory network does not influence the ethanol-induced expression of *exaA3*, the ethanol metabolism should proceed. Deletion of any element of the network even increased expression of *exaA3*. Therefore, it must be postulated that the network also regulates expression of additional genes coding for components of the ethanol oxidation system, similar to what is seen in *P. aeruginosa* (Gliese et al. 2004).

The ethanol-induced expression of *exaS*, *elmS*, and *gacA* was also observed under microaerobic conditions, in which *Azoarcus* sp. strain BH72 is capable of fixing nitrogen (Hurek et al. 1987). Expression of these genes was also detectable in the rhizoplane or even inside rice roots. Additionally, disruption of one of the genes

reduced the competitiveness in colonizing rice roots to the same extent as the *exaA2* deletion mutant (Krause et al. 2011). Therefore, it could be hypothesized that the proposed regulatory network is also regulating in planta the gene expression of *exaA2*. Furthermore, the ethanol-triggered expression of the four *tcs* genes indicates that ethanol is most likely provided by the plant partner of this endophytic interaction. This assumption is corroborated by the results on the *exaA2* and *exaA3* expression as previously described by Krause et al. (2011). Thus, ethanol is an important carbon source for the endophytic life of *Azoarcus* sp. strain BH72.

MATERIALS AND METHODS

Bacterial strains and conditions of cultivation.

Bacterial strains used are listed in Supplementary Table S1. *Escherichia coli* was cultivated at 37°C in Luria-Bertani medium containing suitable antibiotics (Sambrook et al. 1989), while *Azoarcus* sp. strains were generally grown at 37°C in the complex Voll medium (VM) (carbon source as shown below, 3 g of bacto pepton, 1 g of yeast extract, 1.1 g of NaCl, 0.5 g of NH₄Cl, 0.6 g of K₂HPO₄, 0.4 g of KH₂PO₄, 0.2 g of MgSO₄, 66 mg of Fe(III)-EDTA, 26 mg of CaCl₂·2H₂O, 10 mg of MnSO₄·H₂O, 2 mg of Na₂MoO₄·2H₂O, pH 6.8). In addition, the *Azoarcus* strains were cultured aerobically in the synthetic medium (SM) (carbon source as shown below, 0.5 g of NH₄Cl, 0.6 g of K₂HPO₄, 0.4 g of KH₂PO₄, 0.2 g of MgSO₄, 0.1 g of NaCl, 66 mg of Fe(III)-EDTA, 26 mg of CaCl₂·2H₂O, 10 mg of MnSO₄·H₂O, 2 mg of Na₂MoO₄·2H₂O, pH 6.8). Nitrogen-fixing conditions were achieved by using SM devoid of nitrogen in rubber stopper-sealed Erlenmeyer flasks with microaerobic conditions of an initial 1.2% O₂ in the N₂ headspace (Krause et al. 2011). All media were supplemented either with ethanol (6 ml/liter) or malic acid (5 g/liter) as carbon source. Antibiotics used were kanamycin (Km) or Sp at a final concentration of 30 and 40 µg/ml, respectively.

Nucleic acid manipulation and sequence data analysis.

Standard molecular DNA techniques were carried out as described by Sambrook et al. (1989). Sequences were determined

Fig. 4. Detection of bacterial gene expression in the plant-rhizosphere interface. Rice seedlings were inoculated with BH*exaStd*, BH*elmStd*, or BH*gacAtd* and were cultivated gnotobiotically in a growth chamber for 14 days. Bacterial cultures were grown overnight in Voll medium supplemented with ethanol or malate as carbon source. Representative photographs are shown. Settings of the fluorescence microscope and the video camera were equal for all micrographs of roots. Also, for bacterial cultures, all settings were identical.

by the traditional Sanger method (LGC Genomics, Berlin). Total RNA isolation from 20 to 50 ml of overnight cultures grown in complex media with the addition of ethanol were performed using the hot phenol extraction methods as described in more detail by Hauberg-Lotte et al. (2012). Genomic DNA contaminations in the RNA preparation were removed, either by treatment with DNase I (Roche Diagnostics GmbH, Mannheim, Germany) including the RNase inhibitor Superase (Life Technologies, Darmstadt, Germany,) according to manufacturer's instructions and subsequent phenol-chloroform-isoamylalcohol extraction followed by a precipitation step, or by applying the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Finally, RNA samples were stored in 1× RNAsecure (Life Technologies, Darmstadt, Germany).

Analyses of sequences were performed as follows: comparison through the BLAST program (Altschul et al. 1997) of the National Center for Biotechnology Information (NCBI), identification of protein domains through the conserved domain database at NCBI (Marchler-Bauer et al. 2011), the Pfam database of the Sanger Institute (Finn et al. 2014), or TIGRFAM of the J. Craig Venter Institute (Haft et al. 2013), recognition of signal peptides and transmembrane helices through SignalP 3.0 (Bendtsen et al. 2004) or TMHMM 2.0 (Krogh et al. 2001) of the Center for Biological Sequence Analysis (Aarhus, Denmark), and prediction of σ^{70} promoters through BPROM of Softberry, Inc. (Mount Kisco, NY, U.S.A.). Synteny of the *exaA2-exaA3* gene cluster in microbial genomes was analyzed with SyntTax (Oberto 2013) and MultiGeneBlast (Medema et al. 2013).

Construction of mutant strains.

elmS and *gacA* were disrupted either by in-frame deletion or marker exchange mutagenesis. For this, both flanking regions of each gene were amplified using the *Pfu* DNA polymerase (Thermo Fisher Scientific, Sankt Leon-Rot, Germany). The PCR fragments contained 128 bp of the 5' end and 163 bp of the 3' end of *elmS* or, for *gacA*, 71 and 34 bp, respectively. These fragments were cloned into pUC18 or pJET1.2/blunt (Thermo Fisher Scientific) and were subsequently verified by sequencing (LGC Genomics). Both flanking regions of one gene were joined together and were cloned into the mobilizable vector pK18mobsacB (Schäfer et al. 1994). The corresponding plasmids were transferred into *Azoarcus* sp. strain BH72 by biparental conjugation. In a first step, plasmid integration into the chromosome was monitored by the vector-based Km^r. One of these transconjugants was cultivated and, finally, double crossover events were identified by sucrose selection and Km sensitivity. Accuracy of the deletion was then verified by PCR. In order to create the marker exchange mutants, the recombinant pK18mobsacB plasmids carrying the two flanking regions were used. The antibiotic resistance cassette Sm^r/Sp^r obtained from the plasmid pH45Ω (Prentki and Krisch 1984) was cloned into the junction between these two regions. After biparental conjugation into *Azoarcus* sp. strain BH72, selection of double crossovers was performed by the Sm resistance and Km sensitivity. The DNA segment of *exaRS* to be deleted covered 911 bp of the 3' end of *exaS* as well as 558 bp of the 5' end of *exaR*. The deleted fragment was replaced by the Sm^r/Sp^r antibiotic resistance cassette. Suitable DNA fragments around *exaRS* were amplified using the *Pfu* DNA polymerase, followed by cloning into the pK18mob2 (Kirchner and Tauch 2003), verification by sequencing, insertion of the resistant cassette, and conjugation into *Azoarcus* sp. strain BH72. Double crossover events at homologous sites were selected as described above.

To generate transcriptional fusions to *gfp-gusA*, *tdTomato*, or *tdTomato-gusA*, appropriate regions of the 5' end of the desired genes were amplified with DreamTaq DNA polymerase

(Thermo Fisher Scientific). Thereafter, the PCR products were cloned into vectors pK18GGST (Krause et al. 2011), pK18TST (Sarkar et al. 2017), or pK18TGST, the latter two derivatives of pK18GGST in which the *gfp-gusA* cassette was replaced by the promoterless *tdTomato* or by the promoterless *tdTomato-gusA* marker genes. Transfer of the obtained plasmids into *Azoarcus* sp. strain BH72 and its in-frame deletion mutants was performed via biparental conjugation followed by selection of the plasmid integration into the chromosome through vector-based Km^r. Correct integration events at homologous sites of all mutant strains were confirmed by PCR and Southern blot analysis.

Bacterial growth analyses in culture.

The growth of *Azoarcus* sp. strain BH72 and its mutants was determined essentially as described by Krause et al. (2011). For that, strains were precultured in complex VM with ethanol or malate for at least 13 h at 37°C. After centrifugation, cells were washed two times and, finally, were adjusted to an optical density at 600 nm (OD_{600nm}) of 0.05 in SM supplemented either with ethanol or malate. Incubation of the cultures took place at 37°C until stationary phase was reached, during which the OD_{600nm} was determined at different time points. Growth rates were calculated for each culture separately, were averaged, and then, were statistically examined by a two-tailed *t* test.

Quantification of gene expression using the β-glucuronidase activity.

Cells of overnight cultures grown at 37°C in complex VM with malate were collected by centrifugation and were washed two times with nitrogen-free SM containing no carbon source. Finally, they were resuspended in SM for aerobic growth or in nitrogen-free SM for microaerobic growth comprising ethanol or malate as carbon source. Depending on their growth capability in the medium used, cultures were adjusted to an OD_{600nm} of 0.1 or 0.3 and were then incubated for 6 h under aerobic or nitrogen-fixing microaerobic conditions at 37°C. Activity of the β-glucuronidase was assayed quantitatively, as described by Jefferson et al. (1986) with modifications (Krause et al. 2011), and was expressed in Miller units. The average of each culture was calculated and the significance was determined through the two-tailed *t* test.

Analyses of gene expression using RT-PCR.

Semiquantitative RT-PCR was performed from isolated total RNA in a two-step system, according to manufacturer's protocols. In the first step, 80 ng of RNA was applied to cDNA synthesis at 60°C for 30 min with gene-specific reversed primers, using the RevertAid Premium reverse transcriptase (Thermo Fisher Scientific). Thereafter, in the second step, PCR amplification of *exaA2* or *exaA3* with the gene-specific primer sets *exaA2RT-for* (5' CCTCCATCGTCGCTCGAC 3') and *exaA2RT-rev* (5' ACCTTGCCGGTGGGGAGTT 3') or *exaA3RT-for* (5' GGCCAGGAATCGCAACCGCT 3') and *exaA3RT-rev* (5' TTCCATGCGGCCGACCACAC 3') was carried out. For this, 2 µl of the cDNA was amplified using the DreamTaq DNA polymerase (Thermo Fisher Scientific) in 30 cycles of 1 min at 95°C, 0.5 min at 68.5°C, and 1 min at 72°C. 16S rRNA was utilized to ensure that equal amounts of RNA were applied. For this, only 20 ng of RNA was inserted in the cDNA synthesis. The gene-specific primer pairs were either TH3 and TH5 (Hurek et al. 1993) or 799f (Chelius and Triplett 2001) and 1492r (Lane 1991). PCR amplification was performed for 30 cycles at 95°C for 1 min, 70°C for 1 min (TH3-TH5) or 50°C for 30 s (799f-1492r), and 72°C for 1 min. Lack of DNA in the RNA preparation was confirmed by PCR without previous cDNA synthesis. Aliquots (5 µl) were taken at different cycles and were subsequently analyzed on 1.8% agarose gels.

Evaluation of *Oryza sativa* root colonization.

Caryopses of *Oryza sativa* subsp. *indica* cv. IR36 obtained from the International Rice Research Institute (Los Banos, The Philippines) were dehusked, surface sterilized, and germinated as described earlier (Hurek et al. 1994; Krause et al. 2011), with the modification that germination was performed for 3 days in darkness at 30°C, followed by 1 day at 23°C with a daytime of 10 h and a light intensity of approximately 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. Bacterial strains were grown overnight in SM at 37°C, followed by two centrifugation steps with a final resuspension in ethanol-free plant medium (0.6 g of K_2HPO_4 , 0.4 g of KH_2PO_4 , 200 mg of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 100 mg of NaCl, 26 mg of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 20 mg of DL-malic acid, 13 mg of Fe(III)-citrate hydrate, 12 mg of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 4 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 3 mg of H_3BO_3 , 0.2 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, pH 6.8 [Egener et al. 1999]), and 2×10^8 cells were added to the germinated rice seedlings. For the competitive approach, the applied bacterial cells constituted a 1:1 mixture of the wild type and a mutant strain, while, for in planta expression analyses, only single strains were used. In order to verify the accuracy of the 1:1 mixture, dilutions were plated on SM. Then, randomly, 100 colonies were transferred in parallel to VM-ethanol plates, with and without appropriate antibiotics to distinguish the mutant strain from the wild type. Gnotobiotic cultivation of plants under conditions devoid of the addition of external ethanol and quantification of endophytic colonization and competitiveness of mutant strains versus wild type were carried out as described by Krause et al. (2011). Results were statistically analyzed by the two-tailed *t* test.

Before monitoring of *tdTomato* expression, root samples were incubated for 1 h at 4°C. Visualization of *tdTomato* fluorescence was done through the Zeiss Axioplan 2 microscope (Carl Zeiss AG, Jena, Germany), with an excitation filter BP 550/25, a beam splitter FT 570, and an emission filter BP 605/70 (Filter set 43 HE). The filter set 09 (excitation filter BP 450–490, beam splitter FT 510, and emission filter LP 515) was used to monitor root autofluorescence. Micrographs were recorded at identical settings, for comparative pictures, with the Axiocam 503 color camera (Carl Zeiss AG) for both filter sets, and finally, were overlaid.

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