Motility, adhesion and c-di-GMP levels influence the endophytic colonization of rice by *Azoarcus* sp. CIB

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Abstract

Background: Proficient crop production is needed to ensure the feeding of a growing global population. The association of bacteria with plants plays an important role in the health state of the plants contributing to the increase of agricultural production. Endophytic bacteria are ubiquitous in most plant species providing, in most cases, plant promotion properties. However, the knowledge on the genetic determinants involved in the colonization of plants by endophytic bacteria are still poorly understood.

Results: In this work we have used a genetic approach based on the construction of *fliM*, *pilX* and *eps* knockout mutants to show that the motility mediated by a functional flagellum and the *pili* type IV, and the adhesion modulated by exopolysaccharides are required for the efficient colonization of rice roots by the endophyte *Azoarcus* sp. CIB. Moreover, we have demonstrated that expression of an exogenous diguanylate cyclase or phosphodiesterase, which causes either an increase or decrease of the intracellular levels of the second messenger cyclic di-GMP (c-di-GMP), respectively, leads to a reduction of the ability of *Azoarcus* sp. CIB to colonize rice plants.

Conclusions: Here we present results demonstrating the unprecedented role of the universal second messenger cyclic-di-GMP in plant colonization by an endophytic bacterium, *Azoarcus* sp. CIB. The involvement of bacterial motility and adherence mediated by the bacterial flagellum, *pili* type IV and exopolysaccharides in the colonization of rice by strain CIB is also shown. These studies pave the way to further strategies to modulate the interaction of endophytes with their target plant hosts.

Keywords: flagella, pili type IV, c-di-GMP, Azoarcus, endophyte, plant bacteria interaction

Background

Plant associated bacteria play a fundamental role in the healthy state of the plant contributing to its nutrition, development and defence [1-3]. A good number of the associated bacteria colonize the plant tissues as endophytes being roots a major target for endophytic colonization [2]. The effective root colonization by endophytic bacteria involves the coordinated expression of a number of gene factors, many of which are still poorly understood [4-7]. Several comparative studies of the genomes of endophytic bacteria were performed trying to establish the genes involved in plant colonization [2, 8-11]. Hardoim *et al.* [9] compared 40 genomes of endophytic bacteria with genomes of bacterial plant pathogens, bacteria that colonize the rhizosphere and bacteria from the rhizobia group, in order to establish the determinants that differentiate endophytes from non-endophytic bacteria able to interact with plants. Some properties such as motility, chemotaxis and adherence were considered crucial for initiating endophytic colonization. Endophytes are attracted to the root by plant exudates [2], therefore, bacterial motility plays an important role in plant colonization and, in fact, most of the known endophytes have flagella [11]. Chemotaxis towards the plant varies greatly between the different species of endophytes according to the rank of their preferred host [8]. For example, while in *Gluconacetobacter diazotrophicus* only 12 genes related to transmembrane receptors, and two-component response regulators are involved in the signal transduction for chemotaxis, the genome of *Azoarcus* sp. BH72 has 51 such genes, a number similar to that of *Burkholderia phytofirmans* PsJN [11].

The flagellum is considered one of the first bacterial factors that come into contact with the plant, activating the induced systemic resistance [12]. It has been reported that the *Azoarcus* sp. BH72 flagellum has an active role during the effective endophytic colonization of rice roots [13]. Once the bacteria contacted with plant roots, adherence and motility of the twitching type mediated by the *pili* type IV becomes especially important [8, 14]. Thus, the presence of functional *pili* seems to be also essential for the colonization of rice by *Azoarcus* sp. BH72 [4, 15]. After the adhesion of the bacteria to the roots microcolonies are formed involving the essential participation of exopolysaccharides and lipopolysaccharides [16-18], as it has been reported in the colonization of rice roots by *Herbaspirillum seropedicae* [19] and *G. diazotrophicus* [20]. In fact, bacterial exopolysaccharide is related with a multitude of vital functions for the cell, supporting the cohesion and adhesion to biotic and abiotic surfaces through cell aggregation and participating in biofilm formation [21]. In fact, some reports have suggested the participation of exopolysaccharide in the establishment of symbiotic and plant-bacterial interactions between rhizobia and legumes [22], and in the rhizosphere interactions of some strains of *P. fluorescens* [23] or endophytes such *G. diazotrophicus* [20].

Cyclic di-GMP (c-di-GMP) is a second universal messenger in prokaryotes that controls many bacterial processes such as the transition from a planktonic to a sessile state favouring the establishment of biofilms and modulating bacterial virulence [24, 25]. In fact, it has been reported that the levels of c-di-GMP in bacteria control the synthesis of exopolysaccharides, adhesins and the formation of biofilms [26-30], hence regulating, among others, colony morphology [31, 32], quorum sensing processes [33], cell motility [34-37], nodulation [30, 38] and virulence [39, 40]. However, reports on the role of c-di-GMP in plant-bacteria interactions are scarce. The few available studies

have mainly focused on the regulation of virulence in plant pathogenic bacteria [41] such as *Pseudomonas syringae* [42, 43], *P. savastanoi* [42], *Xanthomonas campestris* [44-46] or *X. oryzae* [47, 48], and in the study of the interaction between plants and symbiotic bacteria [42, 49]. The results obtained from these studies are disparate. Thus, whereas increasing c-di-GMP levels by the overexpression of the diguanylate cyclase (DGC) PleD in pathogenic strains *P. syringae* pv. *tomato* and *P. syringae* pv. *phaseolicola* reduces the expression of the type III secretion system (associated with pathogenesis) without affecting virulence, the overexpression of PleD in *P. savastanoi* does not affect the expression of the secretion system but delays the appearance of necrosis [42]. In general, high concentrations of cyclic di-GMP promote biofilm formation, aggregation, attachment, and reduced motility of pathogens such as *Vibrio cholerae* [50], *Xanthomonas campestris* pv. *campestris* [51] or *Yersinia pestis* [52]. In symbionts, the study of how c-di-GMP levels affect interactions, for example between *Rhizobium etli* and *Phaseolus vulgaris* or between *R. leguminosarum* and *Vicia sativa*, have shown that, in both cases, high levels of c-di-GMP favour adhesion to the roots of the plant, associated with a greater formation of biofilm, but negatively affect later stages, reducing nitrogen fixation and, consequently, plant growth [42]. Nevertheless, and as far as we know, there are no reports on the role of c-di-GMP in plant colonization by endophytes.

Azoarcus sp. CIB is a beta-proteobacterium, facultative anaerobic (denitrifying) strain able to colonize rice roots as an endophyte [53] that display plant growth promoting traits such as the ability to fix nitrogen, solubilize inorganic phosphate, produce the plant growth hormone indoleacetic acid [53] and promote the rice plant growth under certain environmental stress conditions [54]. Some genetic determinants likely involved in the endophytic interaction, such as the presence of flagella and *pili*

type IV, can be inferred from the genome sequence of strain CIB [55]. In addition, strain CIB owns some other interesting biotechnological properties such as the ability to degrade toxic aromatic compounds, *e.g.* toluene/xylene, under aerobic and anaerobic conditions [56, 57], and tolerate high concentrations of certain metals and metalloids for their conversion into metal nanoparticles of industrial value [58, 59]. All these features make strain CIB significantly different to the typical *Azoarcus* endophytic strains, and more closely related to members of the new genus *Aromatoleum* [60].

The aim of this work is to explore the role of the flagellum, *pili* type IV and exopolysaccharides on the colonization of rice roots by *Azoarcus* sp. CIB. Since the second messenger c-di-GMP is connected with motility and adhesion, in this work we investigated if the levels of c-di-GMP also control the ability of the bacterium to colonize plant, an aspect scarcely explored in endophytes.

Results and Discussion

A functional flagellum and *pili* type IV are required for rice colonization of *Azoarcus* sp. CIB

As mentioned in the Introduction, bacterial motility plays an important role in plant colonization. The flagellum has traditionally been regarded as the main responsible for bacterial motility. Three gene clusters predicted to control the synthesis and regulation of the flagellum have been identified at three different positions of the genome of *Azoarcus* sp. CIB [55, Supplementary Table S1 in Additional file 1]. We then checked whether a mutation that abolish flagellum functionality may have an effect on the endophytic colonization of rice by strain CIB.

To try to generate motility minus phenotype in *Azoarcus* sp. CIB, we constructed the mutant strain *Azoarcus* sp. CIBd*fliM* (Table 1). This strain harbors a disruptional insertion of the *fliM* (AzCIB_0962) gene, encoding the FliM protein of the C ring which has been shown to be directly involved in flagellum movement and the flagellar export apparatus [61-64]. To check whether the *fliM* mutant strain showed an altered motility phenotype, we performed bacterial swimming assays. As shown in figure 1a, the movement of the mutant cells was significantly diminished (around 50%) with respect to that of the parental strain, hence suggesting an important loss of functionality of the flagellum in *Azoarcus* sp. CIBd*fliM* strain. We then checked the ability of the mutant strain to colonize rice seedlings. Interestingly, the number of endophytes recovered after inoculation of rice seedlings with *Azoarcus* sp. CIBd*fliM* strain was around an order of magnitude lower (3.4×10^3 per g of roots) than those recovered from seedlings inoculated with the wild type CIB strain (4.5×10^4 per g of roots) (Fig. 1b). These results reveal an important role of the flagellum at some stage of the endophytic colonization of rice by *Azoarcus* sp. CIB. These results are in agreement with previous reports showing that deletion of the *fliC3* gene, encoding the main flagellin (FliC) of the flagellum, in the endophyte *Azoarcus* sp. BH72 strain leads to a significant decrease of around 80% in the plant colonizing capacity on competition assays between the wild type and the mutant strain [13], hence supporting that the flagellum is involved in the *Azoarcus*-plant interaction.

Different studies conducted on *Azoarcus* spp. strains have concluded that *pili* type IV are needed for plant colonization since they are responsible of adhesion and movement to the plant roots [4, 13, 15, 65]. The genes predicted to be responsible for the synthesis of *pili* type IV in *Azoarcus* sp. CIB are organized in several genes clusters distributed along the genome [55, Supplementary Table S1 in Additional file 1]. In

order to analyze if the minor pilin PilX from *Azoarcus* sp. CIB is involved in the colonization of rice root, we constructed an *Azoarcus* sp. CIB*dpilX* mutant strain containing a disruption of the *pilX* gene (AzCIB_3117) (Table 1). The motility (swarming pattern) of the *pilX* mutant strain was reduced by 40% compared to that observed with the wild-type strain (Fig. 2a), which is in agreement with the already observed lower motility in *pil* mutant bacterial strains [66, 67] and confirms that the *pil* genes indeed encode proteins that participate in the motility apparatus in *Azoarcus* sp. CIB. Interestingly, the plant colonization ability of the *Azoarcus* sp. CIB*dpilX* strain was more than one order of magnitude lower than that of the parental CIB strain (Fig. 2b). Therefore, these results allowed us to conclude that *pili* type IV are involved in the interaction of the CIB strain with rice. Our results in strain CIB are in agreement with those reported in *Azoarcus* sp. BH72 where a deletion of *pilX* did not inhibit the synthesis of the *pili* but affected its functionality [68, 69] and decreased by 80% the plant colonizing capacity of the mutant strain [4, 15].

In summary, all these results taken together indicate that both, flagellum and *pili* type IV are needed for the interaction of *Azoarcus* sp. CIB with rice.

Role of the genes involved in exopolysaccharide modification in the interaction between *Azoarcus* sp. CIB and rice

As mentioned in the Introduction, exopolysaccharides have an important role in plant-bacteria interactions [20, 22, 23]. In the genome of *Azoarcus* sp. CIB we have identified three gene clusters putatively involved in the synthesis and modification of exopolysaccharides [55, Supplementary Table S1 in Additional file 1]. Previous results have shown that the gene cluster AzCIB_0813-0833 was strongly induced at the transcriptional level in the presence of rice extracts [54]. To analyze the potential role of

this gene cluster in the colonization of rice roots by the CIB strain, it was inactivated by insertional disruption at the *AzCIB_0818 (epsF)* gene. The surface of the colonies of the *Azoarcus* sp. CIB*depsF* mutant strain grown on a solid culture medium supplemented with Congo Red dye [70] showed a rugose phenotype in contrast to the smooth phenotype shown by colonies of the wild-type strain (Fig. 3a). These changes in colony morphology strongly suggest the existence of significant alterations in the exopolysaccharide of the *epsF* mutant strain [71], hence indicating that the *eps* genes are indeed involved in exopolysaccharide synthesis/modification. The next step was to study the ability of the strain *Azoarcus* sp. CIB*depsF* to colonize rice roots as an endophyte. The plant colonization results showed that the number of *Azoarcus* sp. CIB*depsF* endophytes recovered from rice roots (1.65×10^4 bacteria per g of roots) was 36% lower than that obtained from roots inoculated with the parental strain *Azoarcus* sp. CIB (4.57×10^4 bacteria per g of roots) (Fig. 3b). Therefore, the results obtained reveal that the inactivation of the *eps* cluster reduces the ability of *Azoarcus* sp. CIB to interact with the rice plant. These results provide strong experimental support on the role of the exopolysaccharide(s) promoting the endophytic lifestyle in members of the *Azoarcus/Aromatoleum* group.

c-di-GMP levels influence the endophytic lifestyle of *Azoarcus* sp. CIB

As mentioned in the Introduction, c-di-GMP is a second messenger involved in the regulation of many bacterial functions. However, its role in beneficial plant-bacteria interactions and, more specifically, in their endophytic relationships, is poorly studied and far to be understood. Since the production and/or functionality of *pili* type IV, flagellum and exopolysaccharide (all of which were shown above to be involved in the *Azoarcus* sp. CIB-rice interactions) have been related with the intracellular levels of c-

di-GMP in bacteria [72-74], it was tempting to speculate that c-di-GMP could be also regulating the endophytic lifestyle of strain CIB. To study further the proposed new function of c-di-GMP in bacterial endophytes, we checked recombinant *Azoarcus* sp. CIB strains containing different intracellular levels of c-di-GMP. To this end, we used *Azoarcus* sp. CIB (pIZ2133), expressing the PA2133 c-di-GMP phosphodiesterase from *P. aeruginosa* PAO1 [26], and *Azoarcus* sp. CIB (pIZ4959), expressing the PP4959 diguanilate cyclase from *P. putida* KT2440 [75], two strains that show reduced or increased levels of c-di-GMP, respectively, with respect to those of the parental strain *Azoarcus* sp. CIB (pIZ1016) [76, Table 1]. To confirm that the two recombinant strains showed the phenotypes anticipated according to the variations in c-di-GMP levels [25], we analyzed first their swarming and swimming motility. As expected, whereas *Azoarcus* sp. CIB (pIZ2133) (reduced c-di-GMP levels) showed an increased swarming and swimming motility, the strain *Azoarcus* sp. CIB (pIZ4959) (increased c-di-GMP levels) showed a reduced swarming and swimming motility with respect to that of the control strain (Fig. 4). The growth rates of the CIB control strain and its derivative strains CIB (pIZ2133) and CIB (pIZ4959) were similar (data not shown), so the observed different motility cannot be attributed to differences in their cell growth. The morphology of the colonies was later analysed and whereas *Azoarcus* sp. CIB (pIZ2133) generated smoother and more plain colonies than the control strain, *Azoarcus* sp. CIB (pIZ4959) showed rough colonies (Fig. 4), which is consistent with a higher exopolysaccharide synthesis [25].

To analyze whether the altered levels of c-di-GMP in *Azoarcus* sp. CIB (pIZ2133) and *Azoarcus* sp. CIB (pIZ4959) strains might influence their plant colonization abilities, rice seedlings colonization tests were performed. As shown in figure 5, the number of endophytes recovered from seedlings inoculated with *Azoarcus*

sp. CIB (pIZ2133) cells (1.46×10^3 bacteria per g of root), was more than one order of magnitude lower than the number of endophytes recovered after inoculation with the CIB control strain (2.5×10^4 bacteria per g of root). On the other hand, inoculation with the strain *Azoarcus* sp. CIB (pIZ4959) led to 7.63×10^3 endophytes per g of root, which represents a loss of efficiency in colonization of 70% with respect to the control strain. Therefore, these data suggest that either the artificial decrease or increase of c-di-GMP levels in *Azoarcus* sp. CIB affect negatively the endophytic colonization of rice by this bacterium. These results are in agreement with previous observations that any modification in the homeostasis of c-di-GMP negatively affects the interactions between the plant and pathogenic or symbiont bacteria [41-43]. These results can be explained by taken into account that both motility and adhesion are needed for successful plant colonization [2]. Thus, an increase in c-di-GMP levels leads to greater adhesion but, at the same time, to a decrease in bacterial motility; conversely, a decrease of c-di-GMP levels enhance bacterial motility but are detrimental for adhesion and formation of microcolonies needed for the entrance of the bacteria to the internal tissues of the plant [2, 77].

To try to determine if the modification of c-di-GMP levels in *Azoarcus* sp. CIB affected the transcription of genes involved in bacterial motility and/or exopolysaccharide formation, an expression analysis of the *pilYI* gene (encodes a protein for the synthesis and stabilization of *pili* type IV), *fliC* gene (encodes a structural protein of the flagellum) and the *epsF* gene (encodes a protein for exopolysaccharide modification) was performed. The results obtained revealed that the expression of the *epsF* gene was not significantly altered in any of the two strains that have modified levels of c-di-GMP, i.e., *Azoarcus* sp. CIB (pIZ2133) and *Azoarcus* sp. CIB (pIZ4959) (Fig. 6). These results suggest that c-di-GMP does not control the exopolysaccharide

genes at the transcriptional level, pointing to a post-transcriptional control, as already described for exopolysaccharide genes in other bacteria [78], as a more likely regulatory mechanism. Similarly, the expression of the *pilYI* and *fliC* genes was not significantly altered in *Azoarcus* sp. CIB (pIZ2133) (reduced c-di-GMP levels) when compared with their expression in the control strain (Fig. 6), suggesting that the increased motility observed in the former strain might be controlled by a c-di-GMP dependent post-transcriptional regulation as already shown in *P. aeruginosa* PAO1 [79]. In contrast, the expression of *pilYI* and *fliC* genes was 3-3.5 times lower in the strain *Azoarcus* sp. CIB (pIZ4959) than in the control strain (Fig. 6), suggesting that the increase of c-di-GMP levels leads to a transcriptional repression of such genes. The repression of the *pil* and *fli* genes in response to elevated cellular levels of c-di-GMP was previously observed in other bacteria such as *V. cholerae* [80, 81], and is in agreement with the reduced motility observed in swarming and swimming assays (Fig. 4). It is known that the c-di-GMP mediated transcriptional regulation of motility genes takes place through the FleQ regulator in *P. aeruginosa* and *P. putida* [34, 82, 83], or through alterations in chemotactic machinery affecting receptors with PilZ domains [84, 85]. Since *Azoarcus* sp. CIB does not have a FleQ ortholog and only a protein with a putative PilZ domain has been identified by genome search, it is currently difficult to predict the signaling cascade involved in the transcriptional control mediated by c-di-GMP on the *pil* and *fli* genes in the CIB strain.

In summary, all the results presented here show for the first time how the modification of intracellular levels of c-di-GMP in an endophytic bacterium affects its ability to colonize the host plant, and they reveal an unnoticed role of c-di-GMP in bacteria controlling the endophytic lifestyle. More work needs to be done to elucidate the signaling cascade that responds to the levels of c-di-GMP in *Azoarcus* sp. CIB.

Conclusions

The results of the present study describe the participation of the motility mediated by the flagellum and *pili* type IV, the adhesion modulated by exopolysaccharides and, remarkably, the unnoticed participation of the intracellular levels of c-di-GMP in the colonization of rice roots by *Azoarcus* sp. CIB. More experiments needs to be developed to understand the complete landscape of molecular determinants involved in the signaling cascade that responds to the levels of c-di-GMP in *Azoarcus* sp. The results of these experiment might be used to set strategies to modulate the interaction of endophytes with their target plant hosts.

Methods

Strains, seeds and plasmids used

The bacterial strains and plasmids used in this work are listed in Table 1. *Azoarcus* sp. strain CIB was deposited in the Spanish Type Culture Collection (CECT #5669). Seeds from *Oryza sativa* L. GLEVA were kindly provided by Castells Seeds Co. (Spain).

Bacterial growth conditions

Azoarcus strains were grown on MC medium (MA basal medium plus trace elements and vitamins). MA basal medium is composed of the following, per liter of distilled water: 0.33 g of KH₂PO₄, 1.2 g of Na₂HPO₄, 0.11 g of NH₄Cl, 0.1 g MgSO₄ x 7H₂O and 0.04 g of CaCl₂ (pH 7.5) supplemented with trace elements (stock solution 100x: 1.5 g of nitrilotriacetic acid, 3 g of MgSO₄ x 7H₂O, 0.5 g of MnSO₄ x 2H₂O, 1 g of NaCl, 0.1 g of FeSO₄ x 7H₂O, 0.18 g of CoSO₄ x 7H₂O, 0.1 g of CaCl₂ x 2H₂O, 0.18 g of ZnSO₄ x 7H₂O, 0.01 g of CuSO₄ x 5H₂O, 0.02 g of KAl(SO₄)₂ x 12H₂O, 0.01 g of H₃BO₃, 0.01 g

of Na₂MoO₄ x 2H₂O, 0.025 g of NiCl₂ x 6H₂O and 0.3 mg of Na₂ScO₃ x 5H₂O (pH 6.5) per liter of deionized water), vitamin solution (stock 1,000x: 20 mg of biotin, 20 mg of folic acid, 10 mg of pyridoxine-HCl, 50 mg of thiamine-HCl x 2H₂O, 50 mg of riboflavin, 50 mg of nicotinic acid, 50 mg of calcium *D*-pantothenic acid, 50 mg of vitamin B12, and 50 mg of *p*-aminobenzoic acid per liter of distilled water) [56]. As carbon source, 0.2 % (w/v) pyruvate was added. When needed, bacterial strains were also grown on a variant of the VM-ethanol rich medium [86] with the following composition (in 1 liter of distilled water): KH₂PO₄ 0.4 g; K₂HPO₄ 0.6 g; NaCl 1.1 g; NH₄Cl 0.5 g; MgSO₄ x 7H₂O 0.2 g; CaCl₂ 26 mg; MnSO₄ 10 mg; Na₂MoO₄ 2 mg; Fe(III)-EDTA 66 mg; yeast extract 1 g; bactopectone 3 g; ethanol 6 ml (pH 6.8). *E. coli* strains were grown in lysogeny broth (LB) medium [87] at 37 °C. When required, kanamycin (Km) or gentamicin (Gm) were added at 50 µg ml⁻¹ or 10 µg ml⁻¹, respectively. The growth of the cultures was monitored by measuring the absorbance at 600 nm (*A*₆₀₀) using a Shimadzu UV-260 spectrophotometer or by counting viable cells. Cell morphology was analyzed with a Nikon OPTIPHOT-2 phase contrast microscope.

Molecular biology techniques

Standard molecular biology techniques were performed as previously described [88]. DNA fragments were purified with Gene-Turbo (BIO101 Systems). Plasmids and PCR products were purified with a High Pure Plasmid and PCR Product Purifications kits (Roche), respectively. Oligonucleotides were supplied by Sigma Co and they are detailed in Supplementary Table S2 in Additional file 1. All cloned inserts and DNA fragments were confirmed by DNA sequencing with fluorescently labeled dideoxynucleotide terminators [89] and AmpliTaq FS DNA polymerase (Applied Biosystems) in an ABI Prism 377 automated DNA sequencer (Applied Biosystems).

Transformations of *E. coli* was carried out by using the RbCl method or by electroporation (Gene Pulser, Bio-Rad) [88]. Transformation of *Azoarcus* sp. CIB was done by biparental conjugation using the strain *E. coli* S17-1 λ *pir* as donor, following a protocol previously established [90] with slight modifications: donor cells were grown to A_{600} of 5 and the receptor CIB strain was grown on MC medium supplemented with pyruvate 0.2%, and concentrated to reach an A_{600} of 35. The transconjugants were selected on MC medium supplemented with 10 mM glutarate plus the corresponding antibiotic.

Construction of *Azoarcus* sp. CIB*pilX*, *Azoarcus* sp. CIB*fliM* and *Azoarcus* sp. CIB*depsF* mutant strains

For insertional disruption of *pilX*, *fliM* and *epsF* genes in the genome of *Azoarcus* sp. CIB we used single homologous recombination with PCR-amplified DNA fragments obtained with the primer pairs 5'*pilX*/3'*pilX*; 5'*fliM*/3'*fliM* and 5'*epsF*/3'*epsF*, respectively (Supplementary Table S2 in Additional file 1). The obtained fragments were double-digested with the appropriate restriction enzymes, generating the pK18mob*pilX*, pK18mob*fliM* and pK18mob*epsF* recombinant plasmids (Table 1). These plasmids were transferred from *E. coli* S17-1 λ *pir* (donor strain) to *Azoarcus* sp. CIB (recipient strain) by biparental filter mating (88), and exconjugants strains *Azoarcus* sp. CIB*pilX*, *Azoarcus* sp. CIB*fliM* and *Azoarcus* sp. CIB*depsF* were isolated on kanamycin-containing MC agar plates harboring 10 mM glutarate as the sole carbon source for counterselection of donor cells. The mutant strains were verified by PCR amplification using an standard oligonucleotide (F24 or R24) flanking the multiple cloning site of the plasmid and a second specific oligonucleotide external to the

fragment used to perform the homologous recombination (Supplementary Table S2 in Additional file 1).

RNA extraction and quantitative reverse transcription-PCR (qRT-PCR) experiments

RNA was purified from bacterial cells grown up to the middle of the exponential phase and resuspended in a solution containing TE buffer (Tris-HCl 10 mM pH 8.0, EDTA 1mM) and lysozyme 50 mg ml⁻¹ (Sigma). Total RNA was obtained using High Pure RNA Isolation Kit (Roche). The DNA was removed with DNase and Removal Treatment Kit (Ambion). The concentration and purity of RNA was spectrophotometrically determined at A_{260} and calculating the A_{260}/A_{280} ratio, respectively. The cDNA was obtained by using the Transcriptor First Strand cDNA Synthesis kit (Roche). Each RT reaction (20 µl) contained 1 µg RNA, 10 U reverse transcriptase, RNase inhibitor 20 U, dNTPS 1 mM and 60 µM random hexamer primers. The standard procedure of cDNA production includes a 10 min incubation at 25 °C followed by 1 cycle of 30 min at 55 °C and another incubation of 5 min at 85 °C using the Mastercycler Gradient equipment (Eppendorf). Then, 1 µl of the obtained cDNA was used as template for the PCR. The cDNA was PCR-amplified using the required oligonucleotides at final concentration of 0.5 µM and 1 U of DNA polymerase I (Biotools) in a final volume of 50 µl. The qRT-PCR reactions were performed in a LightCycle[®]480 II Real-Time PCR Instrument (Roche). The volume of reaction was 20 µl and contained 1 µl cDNA, 0.25 µM oligonucleotides and 10 µl SYBR Green Master Mix (Roche). The *dnaE* gene was used as housekeeping control as reported before [91]. The results are shown as relative quantification using the $\Delta\Delta C_t$ method [92].

Inoculation of rice seedlings with bacteria

Dehulled rice seeds (*O. sativa* L. GLEVA) were surface sterilized by shaking them at 25 °C for 30 min in 30 ml 1% (v/v) sodium hypochlorite. After rinsing them three times for 10 min in sterile water, the seeds were incubated in VM-ethanol for 48 h. The germ-free seedlings were selected for inoculation. The germination of the seeds continued on humidified filter papers for 24 h prior to inoculation with bacterial cells. Bacteria were obtained as described previously [53]. After growth up to mid-exponential phase, bacterial cells were collected by centrifugation, washed with sterile 0.9% NaCl (w/v) solution, resuspended in 1 ml of sterile distilled water, and the cell suspension was inoculated onto the surface of each seedling in aseptic conditions. After inoculation, the seedlings were grown at 25 °C under natural daylight conditions (10 h of light and 14 h of darkness) for 5-10 days.

Recovery and quantification of endophytes

Rice seedlings were collected 5 days after inoculation with bacterial strains. The roots were weighed, proceeding to its surface disinfection by immersion for 3 minutes in a 1% (v/v) solution of sodium hypochlorite and three washes with sterile water. The roots were then homogenized by adding 1 ml of saline (0.85% NaCl) solution to a Potter-Elvehjem [93]. Once the extract was obtained, serial dilutions were made and plated in VM medium plates (1.5% agar) supplemented with kanamycin in order to determine the number of CFUs.

Motility tests

Swimming-type motility assays were performed on semi-solid VM-agar 0.3% medium plates using cultures grown in VM-ethanol liquid medium up to an A_{600} of 0.6. For

swarming motility assays, plates of MC medium with 0.2% pyruvate (w/v) and semi-solid 0.4% agar were used employing cultures grown in liquid MC medium with 0.2% pyruvate (w/v) up to an A_{600} of 0.6. The plates were incubated for 7 days at 30° C and the diameter of the growth halos of the different *Azoarcus* sp. CIB strains was measured.

Colony morphology

The morphological study of the colonies of *Azoarcus* sp. CIB strains was performed on VM medium plates supplemented with 0.5 g l⁻¹ of Congo Red dye (Sigma-Aldrich). The bacteria were grown in VM-ethanol liquid medium up to an A_{600} of 0.6. Subsequently, serial dilutions were made in saline solution (0.85% NaCl) and plated on VM-Congo Red plates. The plates were incubated for 7 days at 30 °C and the morphology of isolated colonies was analyzed with a Leica MZ16FA stereomicroscope.

Sequence Data Analyses

Nucleotide sequence analyses were done at the National Center for Biotechnology Information (NCBI) server (<https://www.ncbi.nlm.nih.gov/>).

Statistical analysis

The data were analysed with the program IBM SPSS statistics 24 using One-way analysis of the variance (One-way ANOVA) to determine significant differences between the mean obtained in the assays performed. The post hoc Bonferroni test was employed to compare the different conditions. Statistical differences were presented as $p < 0.001$ (***), $p < 0.01$ (**) or $p < 0.05$ (*).

Supplementary information

Additional file 1. Includes all Supplementary Tables.

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Authors contribution

H.F.Ll. performed the experiments and participate in data analyses. E.D. and M.C. wrote the manuscript and collaborate in data analyses. All authors discussed and reviewed the manuscript. The authors read and approved the final manuscript.

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Availability of data and materials

Azoarcus sp. strain CIB was deposited in the Spanish Type Culture Collection (CECT #5669). Seeds from *Oryza sativa* L. GLEVA were kindly provided by Castells Seeds Co. (Spain).

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Figure legends

Fig. 1. Swimming and rice colonization phenotypes of the *Azoarcus* sp. CIB and *Azoarcus* sp. CIBd*fliM* strains. **a** Swimming motility of the *Azoarcus* sp. CIB (carrying the control plasmid pSEVA237) and *Azoarcus* sp. CIBd*fliM* strains, and quantification of the diameter of the halos (n=3); the error bars indicate the standard deviation. **b** Quantification of the number of endophytes recovered from rice roots after inoculation with *Azoarcus* sp. CIB (pSEVA237) and *Azoarcus* sp. CIBd*fliM* strains. Graph shows the CFU values per gram of root (fresh weight) from three independent experiments $\pm$ standard deviation. The asterisks show the significance of the differences observed when comparing the two strains and applying the statistical method of analysis of variance ANOVA and Bonferroni test $p < 0.01$ (**); $p < 0.05$ (*).

Fig. 2. Motility and rice colonization phenotypes of the *Azoarcus* sp. CIB and *Azoarcus* sp. CIBd*pilX* strains. **a** Swarming motility of *Azoarcus* sp. CIB (carrying the control plasmid pSEVA237) (left) and *Azoarcus* sp. CIBd*pilX* (right), and quantification of the diameter of the halos (n=3); the error bars indicate the standard deviation. **b** Quantification of the number of endophytes recovered from rice roots after inoculation of rice seedlings with the *Azoarcus* sp. CIB (pSEVA237) and *Azoarcus* sp. CIBd*pilX*. The graph shows the CFU values per gram of root (fresh weight) of three independent

experiments; error bars indicate standard deviation. Asterisks show the significance of the differences observed when comparing the two strains and applying the statistical method of ANOVA analysis of variance and Bonferroni test $p < 0.01$ (**); $p < 0.05$ (*).

Fig. 3. Colony morphology and rice colonization capacity of the *Azoarcus* sp. CIB and *Azoarcus* sp. CIBdepsF strains. **a** Morphology of the colonies of *Azoarcus* sp. CIB (pSEVA237) and *Azoarcus* sp. CIBdepsF strain grown in VM solid medium supplemented with Congo red (0.5 g l^{-1}). The colonies were visualized with a Leica MZ16FA stereomicroscope. **b** Quantification of the number of endophytes recovered from rice roots after inoculation with *Azoarcus* sp. CIB (pSEVA237) and *Azoarcus* sp. CIBdepsF strains. Graph shows the CFU values per gram of root (fresh weight) of three independent experiments $\pm$ standard deviation. The asterisk shows the significance of the differences observed when comparing the two situations and applying the statistical method of ANOVA analysis of variance and Bonferroni test $p < 0.05$ (*).

Fig. 4. Motility and morphology of the colonies of *Azoarcus* sp. CIB strains expressing genes involved in the metabolism of c-di-GMP. The plates were inoculated with *Azoarcus* sp. CIB (pIZ1016) (empty plasmid), *Azoarcus* sp. CIB (pIZ2133) (expressing the PA2133 phosphodiesterase) and *Azoarcus* sp. CIB (pIZ4959) (expressing the PP4959 diguanylate cyclase).

Fig. 5. Rice roots colonization by *Azoarcus* sp. CIB strains producing different levels of c-di-GMP. Rice seedlings were inoculated with *Azoarcus* sp. CIB (pIZ1016) (empty plasmid), *Azoarcus* sp. CIB (pIZ2133) (expressing the PA2133 phosphodiesterase) and *Azoarcus* sp. CIB (pIZ4959) (expressing the PP4959 diguanylate cyclase). Plants were

grown at 25 °C for 5 days and bacteria present inside the roots were determined as described in Methods. The graph shows the CFU values per gram of root (fresh weight) of three independent experiments; error bars represent the standard deviation. Asterisks show the significance of the differences observed when comparing the strains with modified levels of c-di-GMP with respect to the control strain and applying the statistical method of ANOVA analysis of variance and Bonferroni test $p < 0.01$ (**); $p < 0.05$ (*).

Fig. 6. Expression of the *pilYI*, *fliC* and *epsF* genes in *Azoarcus* sp. CIB strains producing different levels of c-di-GMP. The transcript levels of the *pilYI*, *fliC* and *epsF* genes in the *Azoarcus* sp. CIB (pIZ2133) (black columns) and *Azoarcus* sp. CIB (pIZ4959) (white columns) strains grown in VM-ethanol medium for 24 h, were determined by quantitative RT-PCR using *dnaE* gene as internal control, as detailed in Methods. The expression of the genes in each strain is shown relative to their expression in the control strain *Azoarcus* sp. CIB (pIZ1016). The mean value and the standard deviation corresponding to the results of three independent experiments are indicated. Asterisks show the significance of the differences observed when comparing the level of expression of modified strains with respect to wild-type strain applying the statistical method of ANOVA analysis of variance and Bonferroni test $p < 0.01$.

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Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and characteristic(s)	Reference or source
<i>E. coli</i> strains		
DH10B	F', <i>mcrA</i> , Δ (<i>mrr hsdRMS-mcrBC</i>), Φ 80 <i>lacZ</i> Δ M15, Δ <i>lacX74</i> , <i>deoR</i> , <i>recA1</i> , <i>araD139</i> , Δ (<i>ara-leu</i>)7697, <i>galU</i> , <i>galK</i> , <i>rpsL</i> (Sm ^r), <i>endA1</i> , <i>nupG</i>	Life Technologies
S17-1 λ <i>pir</i>	Tp ^r Sm ^r <i>recA thi hsdRM</i> ⁺ RP4::2-Tc::Mu::Km λ <i>pir</i> phage lysogen	[90]
CC118	Δ (<i>ara-leu</i>), <i>araD</i> , Δ <i>lacX7</i> , <i>galE</i> , <i>galK</i> , <i>phoA20</i> , <i>rpoB</i> , <i>thi-1</i> , <i>rpsE</i> , (Sp ^r), (Rf ^r), <i>argE</i> , (Am), <i>recA1</i>	[94]
<i>Azoarcus</i> strains		
CIB	Wild type strain	[56]
CIB <i>dpilX</i>	Km ^R , CIB mutant by insertion in the <i>pilX</i> gene	This work
CIB <i>dfliM</i>	Km ^R , CIB mutant by insertion in the <i>fliM</i> gene	This work
CIB <i>depsF</i>	Km ^R , CIB mutant by insertion in the <i>epsF</i> gene	This work
Plasmids		
pSEVA237	Km ^r , <i>ori</i> pBBR1, harbors the <i>gfp</i> gene under the control of the <i>PlexA</i> promoter	[95]
pIZ1016	Gm ^r , <i>ori</i> pBBR1MCS-5 derivative vector for cloning and expression harboring the <i>Ptac</i> promoter and the <i>lacI</i> gene	[96]
pIZ2133	Gm ^R , pIZ1016 derivative containing the gene PA2133 from <i>P. aeruginosa</i> PAO1 under the control of the <i>Ptac</i> promoter	[76]
pIZ4959	Gm ^R , pIZ1016 derivative containing the gene PP4959 from <i>P. putida</i> KT2440 under the control of the <i>Ptac</i> promoter	[76]
pK18 <i>mob</i>	Km ^R , <i>oriColE1</i> , Mob ⁺ , <i>lacZα</i> , suicide vector for the construction of insertion mutants by homologous recombination	[97]
pK18 <i>mobpilX</i>	Km ^R , pK18 <i>mob</i> derivative that includes a 455 bp <i>pilX</i> <i>HindIII/BamHI</i> internal fragment	This work
pK18 <i>mobfliM</i>	Km ^R , pK18 <i>mob</i> derivative that includes a 512 bp <i>fliM</i> <i>HindIII/BamHI</i> internal fragment	This work
pK18 <i>mobepsF</i>	Km ^R , pK18 <i>mob</i> derivative that includes a 635 bp <i>epsF</i> <i>HindIII/BamHI</i> internal fragment	This work

Figures

Figure 1

Swimming and rice colonization phenotypes of the *Azoarcus* sp. CIB and *Azoarcus* sp. CIBdfliM strains. a Swimming motility of the *Azoarcus* sp. CIB (carrying the control plasmid pSEVA237) and *Azoarcus* sp. CIBdfliM strains, and quantification of the diameter of the halos (n=3); the error bars indicate the standard deviation. b Quantification of the number of endophytes recovered from rice roots after inoculation with *Azoarcus* sp. CIB (pSEVA237) and *Azoarcus* sp. CIBdfliM strains. Graph shows the CFU values per gram of root (fresh weight) from three independent experiments $\pm$ standard deviation. The asterisks show the significance of the differences observed when comparing the two strains and applying the statistical method of analysis of variance ANOVA and Bonferroni test $p < 0.01$ (**); $p < 0.05$ (*).

Figure 2

Motility and rice colonization phenotypes of the *Azoarcus* sp. CIB and *Azoarcus* sp. CIBdpilX strains. a Swarming motility of *Azoarcus* sp. CIB (carrying the control plasmid pSEVA237) (left) and *Azoarcus* sp. CIBdpilX (right), and quantification of the diameter of the halos (n=3); the error bars indicate the standard deviation. b Quantification of the number of endophytes recovered from rice roots after inoculation of rice seedlings with the *Azoarcus* sp. CIB (pSEVA237) and *Azoarcus* sp. CIBdpilX. The graph shows the CFU values per gram of root (fresh weight) of three independent experiments; error bars indicate standard deviation. Asterisks show the significance of the differences observed when comparing the two strains and applying the statistical method of ANOVA analysis of variance and Bonferroni test p < 0.01 (**); p < 0.05 (*).

Figure 3

Colony morphology and rice colonization capacity of the *Azoarcus* sp. CIB and *Azoarcus* sp. CIBdepsF strains. a Morphology of the colonies of *Azoarcus* sp. CIB (pSEVA237) and *Azoarcus* sp. CIBdepsF strain grown in VM solid medium supplemented with Congo red (0.5 g l⁻¹). The colonies were visualized with a Leica MZ16FA stereomicroscope. b Quantification of the number of endophytes recovered from rice roots after inoculation with *Azoarcus* sp. CIB (pSEVA237) and *Azoarcus* sp. CIBdepsF strains. Graph shows the CFU values per gram of root (fresh weight) of three independent experiments $\pm$ standard deviation. The asterisk shows the significance of the differences observed when comparing the two situations and applying the statistical method of ANOVA analysis of variance and Bonferroni test $p < 0.05$ (*).

Figure 4

Motility and morphology of the colonies of *Azoarcus* sp. CIB strains expressing genes involved in the metabolism of c-di-GMP. The plates were inoculated with *Azoarcus* sp. CIB (pIZ1016) (empty plasmid), *Azoarcus* sp. CIB (pIZ2133) (expressing the PA2133 phosphodiesterase) and *Azoarcus* sp. CIB (pIZ4959) (expressing the PP4959 diguanylate cyclase).

Figure 5

Rice roots colonization by *Azoarcus* sp. CIB strains producing different levels of c-di-GMP. Rice seedlings were inoculated with *Azoarcus* sp. CIB (pIZ1016) (empty plasmid), *Azoarcus* sp. CIB (pIZ2133) (expressing the PA2133 phosphodiesterase) and *Azoarcus* sp. CIB (pIZ4959) (expressing the PP4959 diguanylate cyclase). Plants were grown at 25 °C for 5 days and bacteria present inside the roots were determined as described in Methods. The graph shows the CFU values per gram of root (fresh weight) of three independent experiments; error bars represent the standard deviation. Asterisks show the significance of the differences observed when comparing the strains with modified levels of c-di-GMP with respect to the control strain and applying the statistical method of ANOVA analysis of variance and Bonferroni test $p < 0.01$ (**); $p < 0.05$ (*).

Figure 6

Expression of the *pilY1*, *fliC* and *epsF* genes in *Azoarcus* sp. CIB strains producing different levels of c-di-GMP. The transcript levels of the *pilY1*, *fliC* and *epsF* genes in the *Azoarcus* sp. CIB (pIZ2133) (black columns) and *Azoarcus* sp. CIB (pIZ4959) (white columns) strains grown in VM-ethanol medium for 24 h, were determined by quantitative RT-PCR using *dnaE* gene as internal control, as detailed in Methods. The expression of the genes in each strain is shown relative to their expression in the control strain *Azoarcus* sp. CIB (pIZ1016). The mean value and the standard deviation corresponding to the results of three independent experiments are indicated. Asterisks show the significance of the differences observed when comparing the level of expression of modified strains with respect to wild-type strain applying the statistical method of ANOVA analysis of variance and Bonferroni test $p < 0.01$.

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