The Catabolite Repressor/Activator Cra Is a Bridge Connecting Carbon Metabolism and Host Colonization in the Plant Drought Resistance-Promoting Bacterium *Pantoea alhagi* LTYR-11Z

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ABSTRACT Efficient root colonization is a prerequisite for application of plant growth-promoting (PGP) bacteria in improving health and yield of agricultural crops. We have recently identified an endophytic bacterium, *Pantoea alhagi* LTYR-11Z, with multiple PGP properties that effectively colonizes the root system of wheat and improves its growth and drought tolerance. To identify novel regulatory genes required for wheat colonization, we screened an LTYR-11Z transposon (Tn) insertion library and found *cra* to be a colonization-related gene. By using transcriptome (RNA-seq) analysis, we found that transcriptional levels of an *eps* operon, the *ydiV* gene encoding an anti-FlhD₄C₂ factor, and the *yedQ* gene encoding an enzyme for synthesis of cyclic dimeric GMP (c-di-GMP) were significantly downregulated in the Δ *cra* mutant. Further studies demonstrated that Cra directly binds to the promoters of the *eps* operon, *ydiV*, and *yedQ* and activates their expression, thus inhibiting motility and promoting exopolysaccharide (EPS) production and biofilm formation. Consistent with previous findings that Cra plays a role in transcriptional regulation in response to carbon source availability, the activating effects of Cra were much more pronounced when LTYR-11Z was grown within a gluconeogenic environment than when it was grown within a glycolytic environment. We further demonstrate that the ability of LTYR-11Z to colonize wheat roots is modulated by the availability of carbon sources. Altogether, these results uncover a novel strategy utilized by LTYR-11Z to achieve host colonization in response to carbon nutrition in the environment, in which Cra bridges a connection between carbon metabolism and colonization capacity of LTYR-11Z.

IMPORTANCE Rapid and appropriate response to environmental signals is crucial for bacteria to adapt to competitive environments and to establish interactions with their hosts. Efficient colonization and persistence within the host are controlled by various regulatory factors that respond to specific environmental cues. The most common is nutrient availability. In this work, we unraveled the pivotal role of Cra in regulation of colonization ability of *Pantoea alhagi* LTYR-11Z in response to carbon source availability. Moreover, we identified three novel members of the Cra regulon involved in EPS synthesis, regulation of flagellar biosynthesis, and synthesis of c-di-GMP and propose a working model to explain the Cra-mediated regulatory mechanism that links carbon metabolism to host colonization. This study elucidates the regulatory role of Cra in bacterial attachment and colonization of plants, which raises the possibility of extending our studies to other bacteria associated with plant and human health.

KEYWORDS Cra, carbon metabolism, colonization, exopolysaccharides, biofilm, motility, c-di-GMP

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Today, we know that land plants associate with diverse microorganisms residing in rhizosphere soils, plant surfaces, and inner tissues (1, 2). Plant-beneficial bacteria have received increasing attention due to multiple benefits they offer to the hosts, including improving nutrient uptake, preventing pathogen attack, and mitigating abiotic stresses, such as salinity, drought, and extreme temperatures (3, 4). Since roots are the primary site for plant-microbe interaction, most of the previous studies have concentrated on rhizosphere bacterial communities (5, 6). Notably, some rhizospheric bacteria can colonize the surface of plant roots and further transcend the endodermis barrier to invade internal tissues and begin an endophytic lifestyle (7, 8). In recent years, novel endophytic bacteria with the ability to improve plant growth and health have continued to be isolated from natural habitats (9–11). Nevertheless, successful root colonization of crop plants by indigenous bacteria has been considered a prerequisite for their application in improvement of crop performance and yield (8, 12, 13).

Bacterial attachment to plant root surface is an initial stage commonly involved in root colonization (14, 15). While bacterial chemotaxis and motility help planktonic cells to reach a surface, bacterial adhesion often depends on cell surface structures, such as fimbriae, collagen-like proteins, and exopolysaccharides (EPSs) (16, 17). Adherent bacterial populations can aggregate to form biofilms, which are multicellular structures encased in a self-produced matrix that generally consists of EPS, proteins, extracellular DNA, and other environmental factors (18, 19). By embedding themselves into the biofilm matrix, bacteria are protected from harsh environmental conditions and often possess distinct phenotypes compared to their planktonic counterparts, including increased stress resistance and enhanced nutrient uptake (16, 20). Therefore, the formation of biofilms represents a fundamental strategy of both beneficial and pathogenic bacteria to achieve host colonization (12, 16, 21).

Biofilm formation is a complex process under the control of a variety of signaling systems that respond to numerous environmental signals, such as nutrient availability, oxygen, salinity, iron, calcium, and phosphate (16, 22, 23). Previous studies have shown that bacterial motility and EPS production are important targets for biofilm control strategies, and initiation of biofilm formation is often driven by promoting EPS production and suppressing motility (24–26). For instance, in *Escherichia coli* CsgD activates the synthesis of EPS, including cellulose, while it inhibits the expression of genes involved in flagellum formation (25). As well, the ExpR/Sin quorum-sensing system in *Sinorhizobium meliloti* was found to activate EPS production and downregulate the process of motility (27). Moreover, cyclic dimeric GMP (c-di-GMP), one of the most important secondary messengers in bacteria, has been shown to regulate the transition between planktonic and biofilm lifestyles in a variety of bacterial species (16, 28, 29). In general, the increased production of c-di-GMP stimulates the synthesis of biofilm matrix components, including EPS and proteins, and inhibits bacterial motility by affecting flagellum or type IV pilus activities, while low levels of this messenger molecule promote motility and favor dispersion of established biofilms (16, 30, 31). In addition, regulatory factors such as CsgD, BofA, and SuhB were reported to control the motile-sessile transition through modulation of intracellular c-di-GMP levels (25, 28, 32). While the inverse regulation of EPS production and motility during initiation of surface attachment and biofilm formation is widely conserved in animal- and plant-associated bacteria, the underlying mechanisms deployed by bacteria still remain largely unknown.

As the transition of bacteria from planktonic growth to the sessile lifestyle usually occurs in response to changes in the availability of nutrients, carbon metabolism regulators have been found to contribute to the regulation of biofilm formation and host colonization (33–36). In *E. coli* and related species, the carbon storage regulator CsrA, which is involved in regulating stationary-phase metabolism, acts posttranscriptionally to repress glycogen biosynthesis, gluconeogenesis, and biofilm formation, while activating glycolysis, motility, and acetate metabolism (34, 37). In addition, the cyclic AMP receptor protein Crp, a major transcription factor mediating carbon catabolite repression in Gram-negative bacteria, regulates biofilm formation in diverse

TABLE 1 Tn insertion mutants defective in attachment to wheat roots

Mutant	Tn insertion position ^a	Locus tag	Gene	Protein
40	13460	<i>B1H58_00060</i>	<i>pyrD</i>	Dihydroorotate dehydrogenase
52	13460	<i>B1H58_00060</i>	<i>pyrD</i>	Dihydroorotate dehydrogenase
236	13460	<i>B1H58_00060</i>	<i>pyrD</i>	Dihydroorotate dehydrogenase
11	1711675	<i>B1H58_08065</i>	<i>waaG</i>	Glucosyltransferase
29	1711675	<i>B1H58_08065</i>	<i>waaG</i>	Glucosyltransferase
68	1711675	<i>B1H58_08065</i>	<i>waaG</i>	Glucosyltransferase
155	1711675	<i>B1H58_08065</i>	<i>waaG</i>	Glucosyltransferase
168	3511630	Promoter region of <i>B1H58_16720</i>	<i>galU</i>	UTP-glucose-1-phosphate uridylyltransferase
28	758120	<i>B1H58_03575</i>	<i>cra</i>	Catabolite repressor/activator
46	758120	<i>B1H58_03575</i>	<i>cra</i>	Catabolite repressor/activator
191	758120	<i>B1H58_03575</i>	<i>cra</i>	Catabolite repressor/activator

^aTn insertion site determined by searching the *P. alhagi* LTYR-11Z chromosome sequence (GenBank accession no. [CP019706](#)).

bacterial species (38, 39). In enteric bacteria, another global carbon metabolism regulator involved in carbon catabolite repression is the catabolite repressor/activator FruR/Cra, which is a member of the LacI/GalR family and acts oppositely to CsrA, repressing genes involved in glycolysis while inducing those involved in gluconeogenesis (40, 41). Cra's function is cyclic AMP independent but can be inhibited by the glycolytic metabolic intermediates fructose-1-phosphate (F1P) and fructose-1,6-bisphosphate (FBP) (42, 43). In the presence of glycolytic carbon sources (glucose, fructose, etc.), cytoplasmic glycolytic catabolites such as F1P and FBP accumulate, which bind to the inducer-binding domain of Cra and consequently prevent its binding to the target promoters (41, 44). In addition to controlling carbon flux through the dominant metabolic pathways, Cra also regulates virulence in pathogenic bacteria such as *Salmonella enterica* serovar Typhimurium, *Shigella flexneri*, and enterohemorrhagic *E. coli* (EHEC) (41, 42, 45, 46). Until now, however, there has been only one report showing that Cra promotes biofilm formation in *E. coli* by activating transcription of the *csgDEFG* operon involved in curli biosynthesis (47). Whether this is the only role of Cra in biofilm formation in *E. coli* is not known.

In a recent study, we showed that the endophytic bacterium *Pantoea alhagi* LTYR-11Z isolated from the leaves of *Alhagi sparsifolia* colonizes the root system of wheat and enhances its resistance to drought stress (9). Here we found that Cra of *P. alhagi* LTYR-11Z plays a crucial role in regulating its ability to colonize wheat roots in response to carbon source availability. Our results reveal that Cra inversely regulates biofilm formation and motility in *P. alhagi* LTYR-11Z by directly promoting the transcription of the *eps* operon and the *ydiV* gene encoding an anti-FlhD₄C₂ factor. Furthermore, we identified the diguanylate cyclase (DGC) YedQ as a target of Cra to modulate intracellular c-di-GMP levels responsible for regulating the motile-to-sessile transition and thus demonstrate a c-di-GMP-mediated mechanism for Cra regulation of biofilm formation and motility. Our findings unravel a novel mechanism of regulation by which Cra links carbon metabolism to host colonization and shed light on the yet largely unresolved roles of Cra in bacterium-host interactions.

RESULTS

Isolation of mutants defective in attachment to wheat roots. To identify colonization-related genes, a transposon (Tn) insertion library was generated in the wild-type *P. alhagi* strain LTYR-11Z. Three hundred Tn insertion mutants chosen at random were subjected to attachment assays, and 11 mutants were found to show significantly reduced attachment capacity compared with the wild-type strain. Plasmid rescue and subsequent sequence analysis showed that the 11 mutants had Tn insertions in the coding regions of the *waaG*, *pyrD*, and *cra* genes and in the promoter region of an operon involved in the synthesis of UDP-galacturonic acid (UDP-GalA) (Table 1).

The UDP-GalA operon contains three genes encoding UTP-glucose-1-phosphate uridylyltransferase, UDP-glucose 6-dehydrogenase, and UDP-D-glucuronic acid 4-epimerase,

FIG 1 The *P. alhagi* LTYR-11Z *cra* gene is required for attachment and colonization of wheat roots. (A) Attachment and colonization of wheat roots by *P. alhagi* LTYR-11Z wild-type (WT), Δ *cra* mutant, and complemented strains. The strains were inoculated separately into wheat roots, and attachment to roots and colonization of inner root tissues were determined over time. (B) Competition between the WT strain containing pKT100 and the Δ *cra* mutant containing pBBR1MCS-1 for attachment and colonization of wheat roots. The two strains were coinoculated at 1:1 in wheat roots, and attachment and colonization were monitored over time. (C) Growth curves of bacterial strains in LB medium at 30°C. (D) Growth curves of the WT strain and the Δ *cra* mutant in M9 medium supplemented with 0.5% Casamino Acids and 0.4% glucose or pyruvate at 30°C. Data are shown as means $\pm$ standard deviations (SD) from three independent experiments. Statistical significance was determined by Student's *t* test. **, $P \leq 0.01$; ***, $P \leq 0.001$.

which have been reported to be involved in EPS and lipopolysaccharide (LPS) biosynthesis (48). While *waaG* encodes a glucosyltransferase participating in the assembly of the outer core of the LPS (49), *pyrD* encodes a dihydroorotate dehydrogenase involved in pyrimidine biosynthesis (50). The *cra* gene encodes a global transcription factor involved in carbon catabolite repression.

Cra of *P. alhagi* LTYR-11Z is required for the attachment and colonization of wheat roots. In this study, we further pursued the role of Cra in the regulation of wheat root attachment and colonization by *P. alhagi* LTYR-11Z. A chromosomal *cra* deletion mutation (Δ *cra*) was generated in the wild-type LTYR-11Z strain. Root attachment assays showed that the number of the Δ *cra* mutant cells attached to the wheat root surface was significantly lower than that of the wild-type strain, while complementation with a *cra*-expressing plasmid (pKT100-*cra*) restored the attachment capacity of the mutant to the wild-type level (Fig. 1A). To investigate if the difference in attachment to wheat root surface influenced bacterial colonization of internal root tissues, the numbers of endophytic bacterial cells were determined 1, 4, 7 and 10 days postinoculation (dpi) (Fig. 1A). At 1 dpi, the number of endophytic wild-type LTYR-11Z cells was more than 2-fold higher than that of the Δ *cra* mutant cells. This difference remained at 4, 7, and 10 dpi, and complementation of *cra* partially restored the colonization defects of the Δ *cra* mutant. The Δ *cra* mutant was also tested in competition assay with the wild-type strain. When the Δ *cra* mutant carrying plasmid pBBR1MCS-1 (Cm^r) and the wild-type strain carrying plasmid pKT100 (Km^r) were inoculated together at a 1:1 proportion, the wild-type strain exhibited a significantly stronger colonization activity than the Δ *cra* mutant (Fig. 1B). Similar results were obtained when the Δ *cra* mutant

carrying pKT100 and the wild-type strain carrying pBBR1MCS-1 were used in the competition assay at an inoculum of 1:1 (data not shown). The growth of the Δ *cra* mutant and the complemented strain in LB medium was similar to that of the wild-type strain (Fig. 1C). No significant difference was observed in the growth rates between the Δ *cra* mutant and the wild-type strain when grown in M9 medium supplemented with 0.5% (wt/vol) Casamino Acids and 0.4% (wt/vol) single-carbon sources such as glucose, fructose, succinate, and pyruvate (Fig. 1D) (data not shown). These results suggest that the phenotypic alterations in root attachment and colonization by the wild-type strain and the Δ *cra* mutant were not caused by differences in growth rates. Collectively, these data indicate that the global transcription factor Cra plays an important role in the rhizoplane and endophytic colonization of wheat roots by *P. alhagi* LTYR-11Z.

Determination of the Cra regulon by transcriptome analysis. To understand the regulatory role of Cra, we performed transcriptome sequencing (RNA-seq) to examine the global gene expression of the wild-type LTYR-11Z and the Δ *cra* mutant from cultures grown to late exponential phase in LB medium. Compared to wild-type LTYR-11Z results, transcription of 284 genes was altered by 1.5-fold or more ($P < 0.05$) in the Δ *cra* mutant (see Table S1 in the supplemental material). Cra positively regulated 180 of these genes, whereas 104 were negatively controlled. Consistent with previous studies (44, 51), gluconeogenic genes such as *ppsA*, *pckA*, and *icd* were downregulated, while glycolytic genes, including *pfkA*, *pykF*, *fruBKA*, *adhE*, and *gapB*, were upregulated in the Δ *cra* mutant. Then we tried to look for colonization-related genes under the control of Cra. A putative *eps* operon (*B1H58_14485* to *B1H58_14530*) was significantly downregulated in the Δ *cra* mutant (Table S1), suggesting the positive regulation of EPS production by the Cra protein. In addition, a gene encoding the EAL domain protein YdiV homolog (*B1H58_18220*) was downregulated over 2.2-fold in the Δ *cra* mutant. The YdiV protein is known to be an anti-FlhD₄C₂ factor that acts as a negative regulator of flagellar expression in *S. enterica* serovar Typhimurium (52, 53). Thus, decreased transcription of the putative *ydiV* gene in the Δ *cra* mutant suggested a regulatory role of Cra in flagellum biosynthesis. Furthermore, the gene encoding a GGDEF domain protein (*B1H58_18610*) homologous to the DGC YedQ was also significantly downregulated in the Δ *cra* mutant. Since the GGDEF domain of DGCs catalyzes the synthesis of c-di-GMP involved in the regulation of biofilm formation and motility (16), we hypothesized that Cra may also regulate the motile-sessile switch in *P. alhagi* LTYR-11Z through c-di-GMP signaling.

Cra enhances biofilm formation by promoting EPS production. Using AntiSMASH 4.0 (<http://antismash.secondarymetabolites.org/>), we identified a putative EPS biosynthesis gene cluster (*B1H58_14465* to *B1H58_14645*) that is homologous to the colanic acid biosynthetic gene cluster (BGC0000799; 28% of genes show similarity). Since the *eps* operon involved in this gene cluster is significantly downregulated in the Δ *cra* mutant, we hypothesized that Cra might play a role in EPS production. Therefore, EPS production was quantified in the wild-type, Δ *cra*, and complemented strains. As expected, deletion of the *cra* gene resulted in significantly reduced EPS production, which was fully rescued by complementation with plasmid pKT100-*cra* (Fig. 2A). In addition, complementation of *tuaG* (*B1H58_14500*) restored EPS production of the Δ *tuaG* mutant but failed to eliminate the defect of EPS production in the Δ *cra* Δ *tuaG* double mutant (Fig. 2A), further confirming the essential role of Cra in EPS biosynthesis.

To verify the regulatory role of Cra in expression of the *eps* operon, we quantified the expression of the *wza* (*B1H58_14485*) and *tuaG* genes in the wild-type, Δ *cra* mutant, and complemented strains when they were grown to the late exponential phase. As shown in Fig. 2B, quantitative reverse transcription-PCR (qRT-PCR) analysis revealed that the expression of *wza* and *tuaG* required Cra. Moreover, we introduced a single copy of the *P_{eps}::lacZ* transcriptional reporter fusion into the chromosome of the wild-type strain and the Δ *cra* mutant. Deletion of *cra* significantly reduced the activity of the *eps* promoter, which was partially restored by introducing a plasmid expressing *cra* (pBBR1MCS1-*cra*) (Fig. 2C). To further investigate whether Cra regulated the expres-

FIG 2 Cra enhances biofilm formation by positively regulating the expression of the *eps* operon. (A) EPS quantification of *P. alhagi* LTYR-11Z WT, mutants, and complemented strains. (B) qRT-PCR analysis of mRNA levels of *wza* and *tuaG* (two genes of the *eps* operon). The mRNA levels of the target genes were normalized to 16S rRNA levels. (C) β-Galactosidase analyses of the *eps* promoter activities using the transcriptional *P_{eps}::lacZ* chromosomal fusion reporter expressed in the indicated strains. Strains were grown in LB medium. (D) EMSA was performed to analyze interactions between His₆-Cra and the *eps* promoter. Increasing amounts of Cra (0.3, 0.6, 0.9, and 1.2 μM) were used. As negative controls, a 450-bp fragment from the *wza* coding region instead of the *eps* promoter (control A) and bovine serum albumin (BSA) instead of His₆-Cra (control B) were included in the binding assays. (E) Nucleotide sequences of the *eps* promoter region. Putative −35 and −10 elements as well as the transcriptional start site (+1) are shaded. The putative Cra binding site is underlined and compared with the Cra binding consensus sequence given above. Asterisks denote nonconserved positions in the putative Cra binding site. Mutations introduced in the putative Cra binding site are labeled with red letters below the sequence. (F) EMSA was carried out to determine binding of His₆-Cra to the *eps* promoter (WT motif) and the promoter with mutations in the putative Cra binding site (mutant motif). The increasing amounts of Cra used were 0.8 and 1.0 μM. (G) Biofilm formation by the indicated strains was displayed with crystal violet staining as well as quantified using optical density measurement. The results are means ± SD from three independent biological replicates. *, *P* ≤ 0.05; **, *P* ≤ 0.01; ***, *P* ≤ 0.001 (Student's *t* test).

sion of the *eps* operon directly, we examined the interaction between Cra and the *eps* promoter using an electrophoretic mobility shift assay (EMSA). Incubation of a probe harboring the *eps* promoter sequence (−1 to −450 relative to the ATG start codon of the *wza* gene) with His₆-Cra protein led to the formation of DNA-protein complexes (Fig. 2D). The DNA-protein complexes increased in response to more His₆-Cra present in the reaction mixture (Fig. 2D, lanes 2 to 5), whereas a control DNA amplified from the *wza* coding region did not show detectable Cra binding (Fig. 2D, lane 6), indicating specific interactions between His₆-Cra and the *eps* promoter.

As Cra typically binds the consensus sequence RSTGAAWCSNTHHW (42, 54), the upstream region of the *eps* promoter was examined for such a motif. A putative consensus sequence for Cra extending from −420 to −433 bp upstream of the initiation codon of the *wza* open reading frame (ORF) was identified (Fig. 2E). Consistent with previous studies (47, 54), this putative Cra binding consensus sequence shows high nucleotide conservation in the left half-site (the first 7 nucleotides), but poor conservation in the right half-site (5 nonconserved nucleotides in the last 7 nucleotides). To determine if this putative Cra binding site was essential for protein binding, EMSA was also performed using a probe with conserved bases in the putative Cra binding site replaced by same amount of irrelevant bases (Fig. 2F). As shown in Fig. 2F, mutations in the putative Cra binding site abrogated binding of His₆-Cra to the *eps* promoter. Collectively, these results demonstrate that the *eps* operon is positively and directly regulated by Cra in *P. alhagi* LTYR-11Z.

EPSs have been shown to be important for biofilm formation and thus contribute to the colonization process in many plant-beneficial bacteria (15, 55). Tests of biofilm

FIG 3 Cra inhibits motility by positively regulating the expression of *ydiV*, which encodes an anti-FlhD₄C₂ factor. (A) The swimming motility of the indicated strains was tested in 0.3% agar tryptone plates, and the sizes of swimming zones were measured after 10 h of incubation at 22°C. (B) EMSA of His₆-FlhD₄C₂ and the *flhA* promoter in the absence and presence of YdiV. (C) *ydiV* mRNA relative expression levels determined by qRT-PCR. The relative mRNA level was normalized to 16S rRNA. (D) β-Galactosidase activity of the *ydiV* promoter in the indicated strains. Strains were grown in LB medium. (E) Cra (0.25, 0.5, 0.75, and 1.0 μM) EMSA of the *ydiV* promoter region (−1 to −471). (F) Nucleotide sequences of the *ydiV* promoter region (−1 to −471). Two putative Cra binding sites, C1 and C2, are underlined and compared with the Cra binding consensus sequence. Mutations introduced in the putative Cra binding site C2 are shown in red below the promoter sequence. (G) EMSA was performed to determine binding of His₆-Cra (0.4 and 0.8 μM) to the *ydiV* promoter regions corresponding to −196 to −471 (WT C1 motif), −1 to −203 (WT C2 motif), and −1 to −203 with mutations in the putative Cra binding site C2 (mutant C2 motif). **, *P* ≤ 0.01; ***, *P* ≤ 0.001.

formation using a crystal violet staining assay (56) showed that the *Δcra* mutant and the *Δcra ΔtuaG* double mutant form biofilm at significantly lower levels than the wild-type strain (Fig. 2G). Furthermore, complementation of *cra* eliminated the biofilm formation defects of the *Δcra* mutant but failed to restore the biofilm formation ability of the *Δcra ΔtuaG* mutant (Fig. 2G). These results indicate that Cra plays a positive role in biofilm formation by promoting the expression of the *eps* operon.

Cra suppresses motility via positive regulation of *ydiV* expression. Given that the regulatory role of Cra in bacterial motility has never been reported previously, we first examined the motility phenotype in the wild-type, *Δcra* mutant, and complemented strains. Swimming motility assays showed that the *Δcra* mutant was much more motile than the wild-type strain, and the motility was restored to the wild-type levels by complementation with the *cra*-expressing plasmid (pKT100-*cra*) (Fig. 3A). These results clearly indicate that Cra suppresses swimming motility in *P. alhagi* LTYR-11Z.

Although EAL domain proteins are often known to be c-di-GMP-specific phosphodiesterases (PDEs) that degrade c-di-GMP and lead to enhancement of motility in bacteria, the EAL domain protein YdiV has been shown to be an anti-FlhD₄C₂ factor that acts as a posttranscriptional negative regulator of the flagellar master transcriptional activator complex FlhD₄C₂ and inhibits motility (52, 53). To investigate the role of the YdiV homolog (B1H58_18220) in motility, we deleted the *ydiV* gene in the wild-type strain. The *ΔydiV* mutant showed increased swimming motility compared to the wild-type and complemented strains (Fig. 3A). In addition, the *Δcra ΔydiV* double mutant showed increased swimming motility compared to the *Δcra* mutant, and complementation of *cra* failed to restore motility of the *Δcra ΔydiV* mutant to wild-type levels (Fig. 3A). These results suggested that in *P. alhagi* LTYR-11Z, the EAL domain protein YdiV also acts as an anti-FlhD₄C₂ factor. To confirm the anti-FlhD₄C₂ factor activity of YdiV, we examined the effect of His₆-YdiV on the interaction between

His₆-FlhD₄C₂ and the *flhA* promoter using an EMSA. As expected, incubation of purified His₆-FlhD₄C₂ with a 332-bp *flhA* promoter sequence (−4 to −335) led to retarded mobility of the probe (Fig. 3B, lanes 2 to 3), indicating that His₆-FlhD₄C₂ directly binds to the *flhA* promoter. When purified His₆-YdiV was added together with His₆-FlhD₄C₂, the amount of the shifted band corresponding to the FlhD₄C₂-DNA complex decreased and the free probe band appeared (Fig. 3B, lanes 4 to 6). The shifted band disappeared when His₆-YdiV was added at a 4-fold molar excess over His₆-FlhD₄C₂ (Fig. 3B, lane 6). Moreover, addition of His₆-YdiV alone to the reaction mixture gave no shifted band (Fig. 3B, lane 7), indicating that the YdiV protein itself was unable to bind directly to the *flhA* promoter. Collectively, these results indicate that *ydiV* encodes an anti-FlhD₄C₂ factor that inhibits the binding of FlhD₄C₂ to the *flhA* promoter.

Then we sought to determine whether Cra suppresses motility by altering the expression of the *ydiV* gene in *P. alhagi* LTYR-11Z. Consistent with the RNA-seq results, qRT-PCR analysis also revealed that the transcription level of *ydiV* in the Δ *cra* mutant was significantly lower than those in the wild-type and complemented strains (Fig. 3C). The expression of *ydiV* was then investigated by chromosomal *P_{ydiV}::lacZ* fusion reporter analysis. As shown in Fig. 3D, deletion of *cra* significantly reduced the activity of the *ydiV* promoter, which was fully restored upon complementation. To determine whether *ydiV* expression is regulated directly by Cra, an EMSA was performed. Incubation of purified His₆-Cra with a 471-bp *ydiV* promoter region (−1 to −471) led to the formation of protein-DNA complexes in an His₆-Cra concentration-dependent manner (Fig. 3E, lanes 2 to 5), whereas replacement of this *ydiV* promoter probe with an unrelated DNA fragment from the encoding region of *ydiV* abolished the formation of DNA-protein complexes (Fig. 3E, lane 6), indicative of direct binding of His₆-Cra to the *ydiV* promoter.

While two putative promoters were predicted using the BPROM software (Softberry), two putative Cra binding sites, C1 and C2, were identified in the upstream regions of the two putative promoters (Fig. 3F). The two putative Cra binding sequences C1 and C2 in the *ydiV* promoters showed high nucleotide conservation in the left half-site (6 conserved nucleotides in the first 7 nucleotides of the consensus sequence), but poor conservation in the right half-site (4 and 3 nonconserved nucleotides in the last 7 nucleotides, respectively) (Fig. 3F). We then used EMSAs to directly address whether His₆-Cra binds the two putative Cra binding sites. When a 276-bp promoter probe (−196 to −471) containing only the putative binding site C1 was used in the EMSA, the binding of His₆-Cra with this probe was not observed (Fig. 3G), indicating that Cra does not recognize and bind the putative C1 element. In contrast, His₆-Cra was found to bind to the promoter probe (−1 to −203) containing the putative C2 element (Fig. 3G), while mutation of the 7 conserved nucleotides in the first 8 nucleotides of the C2 element abrogated binding of this protein to the probe (Fig. 3G). Collectively, these results demonstrate that Cra activates the expression of *ydiV* by directly binding to its promoter through recognition of the C2 element.

Taken together, our data demonstrate that Cra suppresses motility by directly regulating the expression of the *ydiV* gene, which encodes an anti-FlhD₄C₂ factor of the flagellar regulon in *P. alhagi* LTYR-11Z.

Cra positively regulates the cellular c-di-GMP levels by transcriptional activation of a diguanylate cyclase. As the significantly downregulated gene *B1H58_18610* in the Δ *cra* mutant encodes a GGDEF domain protein homologous to the DGC YedQ, we examined whether this protein possessed DGC activity. Using an *in vitro* enzymatic assay (57), we observed the complete conversion of 20 nmol of GTP to c-di-GMP by 0.7 nmol of purified His₆-B1H58_18610 within 6 h (see Fig. S1 in the supplemental material), clearly indicating that the *B1H58_18610* gene encodes a DGC protein.

Next, we also determined the role of Cra in the expression of *yedQ* (*B1H58_18610*) by qRT-PCR (Fig. 4A) and chromosomal *P_{yedQ}::lacZ* fusion reporter analysis (Fig. 4B), both of which showed that the expression of *yedQ* is positively regulated by Cra in *P. alhagi* LTYR-11Z. In addition, EMSA analysis showed that the purified His₆-Cra protein bound specifically to the *yedQ* promoter probe (−13 to −372) *in vitro* (Fig. 4C). In agreement, analysis of the *yedQ* promoter region revealed a putative Cra binding site

FIG 4 Cra positively regulates the expression of *yedQ*, a diguanylate cyclase-encoding gene, by directly binding to the *yedQ* promoter. (A) *yedQ* mRNA relative expression levels determined by qRT-PCR. The values shown were normalized to the expression level of the 16S rRNA gene. (B) β -Galactosidase activity of a transcriptional fusion of the *yedQ* promoter region to *lacZ* was assayed in the indicated strains. Strains were grown in LB medium. (C) Cra (0.15, 0.3, 0.45, 0.6, and 0.75 μ M) EMSA of the *yedQ* promoter region (–13 to –372). (D) Nucleotide sequences of the *yedQ* promoter region (–1 to –372). The putative Cra binding site is underlined, and mutations in the sequence are labeled with red letters. (E) Cra (0.4 and 0.8 μ M) EMSA of the *yedQ* promoter region (–13 to –372) (WT motif) versus the *yedQ* promoter region with mutations in the putative Cra binding site (mutant motif). (F) Relative intracellular levels of c-di-GMP measured using the transcriptional P_{cra} -*lacZ* reporter. The levels of c-di-GMP in the wild-type strain carrying the vector control pBBR1MCS-1 were set to 1. (G) Swimming abilities of the WT, the Δ *yedQ* mutant, the complemented strain, and the Δ *cra* Δ *yedQ* double mutant. (H) EPS quantification in the indicated strains. (I) The biofilm formation ability of the indicated strains was determined by crystal violet staining. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

highly similar to the Cra binding consensus sequence (Fig. 4D). Arbitrary mutation of the conserved left half-site (GCTGAAGC) of the putative consensus sequence at the –274 to –281 position to CACAGGGA resulted in abrogation of His₆-Cra binding to the probe (Fig. 4E). These results clearly demonstrated that Cra positively regulates *yedQ* expression by directly binding to its promoter.

Furthermore, we compared the levels of c-di-GMP between the wild type and the Δ *cra* mutant by using the P_{cra} -*lacZ* reporter, which responds to the intracellular c-di-GMP concentration (32). As shown in Fig. 4F, the c-di-GMP levels of the Δ *cra* mutant decreased to 48% compared with the wild-type strain, while complementation with the *cra* gene restored the production of c-di-GMP to a level similar to that of the wild-type strain, supporting the positive regulatory role of Cra in intracellular c-di-GMP levels.

FIG 5 Cra regulation is modulated by the availability of carbon sources. (A) β -Galactosidase activities of the *eps*, *ydiV*, and *yedQ* promoters in bacterial strains grown to the stationary phase in M9 medium supplemented with Casamino Acids (0.5%) and the indicated carbon source (0.4%). (B to D) Effects of FBP on Cra binding to the *eps* (B), *ydiV* (C), and *yedQ* (D) promoters. Ten and 50 mM concentrations of FBP were added to 2 ng μ l⁻¹ probe and 700 nM Cra. G6P and F6P were used as negative controls. (E) β -Galactosidase activities of the *eps*, *ydiV*, and *yedQ* promoters in the WT strain and the Δ *pfkA* mutant grown to stationary phase in M9 medium supplemented with 0.5% Casamino Acids and 0.4% glucose. (F) Effects of wheat root exudates on activities of the *eps*, *ydiV*, and *yedQ* promoters in the WT strain and Δ *cra* mutant. Bacterial strains grown in LB medium were harvested, washed, and resuspended in 14-day-old wheat root exudates or the control 1/2 MS medium to give the initial concentration of 10^8 cells ml⁻¹. β -Galactosidase activities were determined after 9 h of incubation at 30°C. Data represent means $\pm$ SD from three biological replicates, each of which was carried out in three technical replicates. *, $P \leq 0.05$; **, $P \leq 0.01$; ns, not significant (Student's *t* test).

To determine the role of *yedQ* in EPS biosynthesis, biofilm formation, and motility, this gene was deleted in the wild-type strain LTYR-11Z. Compared with the wild-type strain, Δ *yedQ* cells swam faster, but the mutant was severely affected in EPS production and biofilm formation (Fig. 4G to I). These phenotypes were restored by a plasmid expressing *yedQ* (Fig. 4G to I), indicating that YedQ plays a role in suppressing motility and enhancing EPS production and biofilm formation. Collectively, these results demonstrate that Cra can regulate EPS biosynthesis, biofilm formation, and motility via transcriptional activation of the DGC YedQ. Deletion of *cra* in the Δ *yedQ* mutant further stimulated motility and led to reduced biofilm formation (Fig. 4G and I), supporting our finding that Cra regulates biofilm formation and motility through more than one pathway.

Regulatory function of Cra is affected on glycolytic and gluconeogenic carbon sources. As the regulatory function of Cra can be modulated by the availability of carbon sources (42, 54), we hereby examined whether the expression of the *eps* operon, *ydiV*, and *yedQ* genes was differentially affected on glycolytic and gluconeogenic carbon sources. In the wild-type LTYR-11Z strain, the activities of the P_{eps} , P_{ydiV} , and P_{yedQ} promoters at 0.4% pyruvate (gluconeogenic) were more than 3-fold higher than those at 0.4% glucose (glycolytic) (Fig. 5A). Moreover, deletion of *cra* almost completely abolished the carbon source-dependent expression of the *eps* operon, *ydiV*, and *yedQ* (Fig. 5A), further supporting the role of Cra in regulation of the *eps* operon, *ydiV*, and *yedQ* in response to carbon source availability.

It has been reported that Cra binding to its targets can be counteracted *in vitro* by micromolar amounts of F1P or millimolar amounts of FBP (42, 58). To assess the role of FBP in the binding of Cra to the promoters of the *eps* operon, *ydiV*, and *yedQ*, EMSAs were performed with 10 and 50 mM FBP, respectively. At a concentration of 700 nM, Cra

completely shifted the promoter probes of *eps*, *ydiV*, and *yedQ* (Fig. 5B to D, lane 2). Addition of 10 mM FBP reduced the intensity of the shifted bands corresponding to the Cra-DNA complexes, resulting in the reappearance of free probe bands (Fig. 5B to D, lane 3). Furthermore, when FBP was added at a concentration of 50 mM, the shifted band almost disappeared (Fig. 5B to D, lane 4). In contrast, fructose-6-phosphate (F6P) and glucose-6-phosphate (G6P) were not able to dissociate the Cra-DNA complexes at the concentration of 50 mM (Fig. 5B to D, lanes 5 and 6). In addition, a previous study in *S. flexneri* had shown that blocking glycolysis by mutation of the *pfkA* gene encoding 6-phosphofructokinase may cause transient accumulation of F6P but a decrease in the concentrations of metabolic intermediates such as FBP (41). Here we show that although deletion of the *pfkA* gene did not affect the growth rate or maximum cell density of *P. alhagi* LTYR-11Z in M9 medium supplemented with 0.4% glucose and 0.5% Casamino Acids (data not shown), it led to significantly increased activities of the *eps*, *ydiV*, and *yedQ* promoters (Fig. 5E). Together, these results support the idea that the binding of Cra to the promoters of the *eps* operon, *ydiV*, and *yedQ* is inhibited by glycolytic metabolic intermediates such as FBP.

Since plant root exudates play important roles in plant-microbe interactions and might regulate the expression of colonization-related genes in plant growth-promoting (PGP) bacteria (5, 13), we further investigated the effects of wheat root exudates on the activities of the *eps*, *ydiV*, and *yedQ* promoters. While root exudates collected from 7-day-old wheat seedlings grown hydroponically in 1/2 Murashige and Skoog (MS) medium without carbon source did not stimulate the activities of the *eps*, *ydiV*, and *yedQ* promoters (data not shown), their activities in the wild-type strain were significantly enhanced after 9 h of incubation in 14-day-old wheat root exudates (Fig. 5F), without a significant increase in the bacterial cell density. In contrast, the 14-day-old wheat root exudates failed to enhance the activities of the *eps*, *ydiV*, and *yedQ* promoters in the Δ *cra* mutant (Fig. 5F). These results suggest that root exudates collected from wheat seedlings at some developmental stages could increase the activating effects of Cra on expression of its target genes.

Effect of carbon sources on attachment of wheat roots by *P. alhagi* LTYR-11Z.

As Cra transcriptionally activates the expression of the *eps* operon, *ydiV*, and *yedQ* genes in response to carbon source availability, we further sought to determine whether glycolytic and gluconeogenic carbon sources affect the attachment capacity of *P. alhagi* LTYR-11Z. *P. alhagi* LTYR-11Z was inoculated with wheat seedlings separately dipped in half-strength MS medium supplemented with 0.4% glucose or pyruvate as the sole carbon source, and the number of bacterial cells attached to the root surfaces was determined. After 1 h of incubation, the number of wild-type cells attached in the presence of 0.4% pyruvate was similar to that in the presence of 0.4% glucose (Fig. 6A). However, 15 h after incubation, the number of the wild-type cells attached in the presence of 0.4% pyruvate reached $3.7 \times 10^8 \pm 1.6 \times 10^8$ CFU g⁻¹ fresh weight, which is over 4-fold higher than that in the presence of 0.4% glucose. Deletion of the *cra* gene reduced wheat root attachment of *P. alhagi* LTYR-11Z under both glycolytic and gluconeogenic conditions, whereas the decrease in attachment capacity was much more pronounced when cells were grown with pyruvate (Fig. 6A). Subsequently, a competition assay was performed with the mCherry-labeled Δ *cra* mutant and the green fluorescent protein (GFP)-labeled wild-type LTYR-11Z at 1:1 in half-strength MS medium supplemented with 0.4% pyruvate. After 15 h of incubation, confocal microscopy observation revealed that the wheat roots were predominantly colonized by the wild-type strain (Fig. 6B). Altogether, these data revealed that Cra plays an important role in regulation of the attachment capacity of *P. alhagi* LTYR-11Z, which can be modulated by carbon availability.

DISCUSSION

In a recent study, we identified a novel endophytic bacterium, *P. alhagi* LTYR-11Z, that can colonize the wheat endorhizosphere and improve plant growth and drought stress tolerance (9). In this work, we performed a small-scale Tn screen and identified

FIG 6 Carbon regulation of the attachment capacity of *P. alhagi* LTYR-11Z. (A) Attachment of wheat roots by the *P. alhagi* LTYR-11Z WT and Δcra mutant after 1 and 15 h of incubation in 1/2 MS medium supplemented with 0.4% glucose or pyruvate as the sole carbon source. Data are shown as means $\pm$ SD from three independent experiments. *, $P \leq 0.05$; **, $P \leq 0.01$; ns, not significant (Student's *t* test). (B) Confocal microscopy analysis of the competition between the GFP-labeled WT and the mCherry-labeled Δcra mutant for wheat root attachment after 15 h of incubation in 1/2 MS medium supplemented with 0.4% pyruvate. The GFP and mCherry fluorescences are visible in green and red, respectively, while the wheat root cells stained with DAPI are visible in blue. Bar, 100 μ m.

3 genes and one operon related to the root colonization capacity of *P. alhagi* LTYR-11Z. Among these colonization-related genes, *waaG* and the UDP-GalA operon encode enzymes involved in the biosynthesis of LPS and EPS, both of which have been known to play an important role in bacterial attachment and colonization of host tissues (14, 15). In addition, *pyrD*, which is involved in pyrimidine biosynthesis, was shown to be required for biofilm formation in *Pseudomonas aeruginosa* (50). While a variety of previous studies have documented the crucial roles of the carbon metabolism regulators CsrA and Crp in host colonization (33, 35, 36), relatively little is known about the regulatory function of Cra in the bacterial colonization process. In this study, we further uncovered a novel carbon source-dependent mechanism controlled by Cra that bridges a connection between carbon metabolism of *P. alhagi* LTYR-11Z and plant colonization.

While the *cra* gene (originally designated *fruR*) is adjacent to the fructose operon *fruBKA* in *Pseudomonas putida* (43), this gene (B1H58_03575) lies far away from *fruBKA* (B1H58_14040 to B1H58_14050) in *P. alhagi* LTYR-11Z, in line with members of enteric bacteria, such as *E. coli* and *Yersinia pseudotuberculosis* (43, 44, 59). It has been reported that carbon nutrition influences colonization and maintenance of pathogenic and commensal *E. coli* strains in the mouse intestine (60–62), while the pathogenic EHEC O157:H7 strain required gluconeogenic nutrients to successfully colonize the intestines precolonized with the commensal *E. coli* Nissle 1917 (63). Given the fact that Cra serves as an activator of gluconeogenic enzyme genes and a repressor of glycolytic enzyme genes, it is not surprising that Cra plays a regulatory role in host colonization and persistence. In agreement, we found that deletion of *cra* attenuates attachment and colonization of *P. alhagi* LTYR-11Z to wheat roots. However, a previous study by Gore and Payne (41) showed the *cra* mutant of *S. flexneri* had an increase in attachment and invasion of Henle cells compared to the wild-type strain, although the exact mechanism

remained unknown. The role of Cra in host colonization seems to be opposite in *P. alhagi* and *S. flexneri*, implying functional differences of Cra in the regulatory networks in different bacterial species.

Carbon metabolism regulators have been shown to regulate biofilm formation via various different mechanisms (34, 37–39). Previous studies in *E. coli* demonstrated that CsrA posttranscriptionally represses the EPS poly-*N*-acetylglucosamine biosynthetic operon *pgaABCD* involved in biofilm formation, while the Crp-cyclic AMP complex and Cra transcriptionally activate *csgD*, which encodes the major regulator of biofilm formation (37, 38, 47). Our results also indicate that Cra is an activator of biofilm formation in *P. alhagi* LTYR-11Z. It should be noted, however, that the genome of this endophytic strain does not contain the *csgD* gene, the target of Crp and Cra in *E. coli* (38, 47), suggesting that in *P. alhagi* LTYR-11Z Crp and Cra regulate biofilm formation via yet unknown mechanisms. In this study, we provide strong evidence demonstrating that Cra enhances EPS biosynthesis through activating transcription of the *eps* operon, thus stimulating biofilm formation in *P. alhagi* LTYR-11Z. Nevertheless, the roles of CsrA and Crp in *P. alhagi* LTYR-11Z biofilm formation remain unresolved and deserve further investigation.

While flagellum-mediated motility can direct bacterial migration toward favorable conditions in response to environmental stimuli (26, 64), the initiation of biofilm formation often leads to the inhibition of flagellar synthesis (25, 65). In fact, in *E. coli* the regulators of biofilm formation, such as CsrA, Crp, and BolA, have been shown to regulate flagellar motility by controlling the expression of the flagellar master operon *flhDC* at the transcriptional or posttranscriptional level (26, 36, 66). In addition, some proteins, such as FlhZ, FlhT, and YdiV, have been shown to act as posttranslational activators or inhibitors of FlhD₄C₂ and thereby regulate the expression of FlhD₄C₂-dependent flagellar genes (52, 53). A previous study in *S. enterica* serovar Typhimurium has shown that the translation, but not transcription, of the *ydiV* gene was enhanced in poor medium compared with that in rich medium, although the underlying mechanisms remained unknown (52). While whether CsrA plays a role in nutritional control of the YdiV expression in *S. enterica* serovar Typhimurium is controversial (52, 67), our results in the present work demonstrate a novel mechanism for modulation of flagellar motility by showing that Cra in *P. alhagi* LTYR-11Z inhibits flagellar synthesis through transcriptionally activating the *ydiV* gene, which encodes an anti-FlhD₄C₂ factor. As Cra plays a role in transcriptional regulation in response to carbon source availability, our finding provides a new insight into how carbon nutrients affect motility.

In recent years, the bacterial second messenger c-di-GMP has received great attention due to its ability to regulate a wide range of cellular processes, including motility, virulence, cell cycle progression, biofilm formation, and dispersal (16, 68). This molecule is synthesized by DGCs with the highly conserved GGDEF domain, while its degradation is carried out by PDEs with an EAL or HD-GYP domain (28). Accordingly, an array of regulatory factors modulate the intracellular concentration of c-di-GMP in bacteria through controlling the expression of DGCs and/or PDEs (25, 28, 32). In *E. coli*, the carbon storage regulator CsrA was shown to control c-di-GMP metabolism by regulating the expression of GGDEF proteins at the posttranscriptional level (69). Here we show that Cra directly regulates the expression of *yedQ* at the transcriptional level and causes the accumulation of c-di-GMP, thus favoring the sessile lifestyle. Hence, our study introduces a novel mechanism of regulation linking carbon metabolism to c-di-GMP signaling. Further studies are warranted to clarify the detailed mechanisms underlying the c-di-GMP-mediated regulation of motility and biofilm formation.

Overall, our results directly connect Cra with the control of motility, EPS production, and c-di-GMP signaling, forming a bridge between carbon metabolism and colonization of host plants. When the carbon source is gluconeogenic, the Cra protein, which has been reported to form a tetrameric complex in *E. coli* (54), activates transcription from the promoters of the *eps* operon and *ydiV* and *yedQ* genes, thereby inhibiting motility and inducing EPS production and biofilm formation. Conversely, within a

FIG 7 Model of the Cra-mediated regulatory mechanism that links carbon metabolism to host colonization in the plant drought resistance-promoting endophytic bacterium *P. alhagi* LTJR-11Z. Under glycolytic conditions, glycolytic catabolites such as FBP bind to the tetrameric Cra protein, decreasing its binding affinity for target promoters. The result is that the expression of the *eps* operon, *ydiV*, and *yedQ* genes is not activated (left panel). Under gluconeogenic conditions, the tetrameric Cra protein activates transcription from the promoters of the *eps* operon, *ydiV*, and *yedQ* genes, which promotes EPS production and biofilm formation and results in reduction in motility (right panel).

glycolytic environment, catabolites such as FBP bind to the tetrameric Cra protein and consequently decrease the activating effect of Cra. Thus, although Cra exerts activating effects on the expression of *eps*, *ydiV*, and *yedQ*, regardless of the carbon source used, the activating effects in medium with the gluconeogenic substrate pyruvate are much stronger than those in medium with the glycolytic substrate glucose. Therefore, the combination of c-di-GMP-dependent and -independent regulatory pathways allows Cra to regulate motility and biofilm formation at various levels and thereby to modulate the switch between a motile and a sessile lifestyle (Fig. 7).

It has been reported that up to 40% of photosynthetically fixed carbon enters the rhizosphere soil via plant roots as labile root exudates, which serve important roles at the soil-root interface as chemical attractants and repellants and thus shape the root microbiome (7, 70, 71). Root exudates consist of an enormous range of compounds, such as sugars, organic acids, amino acids, phytohormones, and complex polymers (70, 72). In addition, the amount and composition of root exudates vary with the plant species and genotype, developmental stage, plant growth substrate, and stress factors (70, 73). Our results suggest that the root exudates from wheat seedlings in a hydroponic culture system may provide gluconeogenic nutrients to facilitate root colonization by activating Cra-mediated regulatory pathways. In the highly competitive environment of the rhizosphere, where diverse bacterial species coexist, bacteria like *P. alhagi* LTJR-11Z may utilize this Cra-mediated strategy to achieve root colonization when gluconeogenic carbon sources are available from root exudates. However, more

in-depth studies are required to validate and further expand our findings. Whether pathogenic and commensal bacteria in the intestine adapt to their particular niche through a similar regulatory mechanism also deserves investigation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 2. The sequences of all primers are listed in Table 3. *E. coli* strains were usually grown in LB medium at 37°C with agitation, and *P. alhagi* LTYR-11Z and its mutants were usually cultivated in LB broth at 30°C, unless specified otherwise. When appropriate, antibiotics were used at the following concentrations: ampicillin, 100 $\mu\text{g ml}^{-1}$; kanamycin, 50 $\mu\text{g ml}^{-1}$; tetracycline, 25 $\mu\text{g ml}^{-1}$; chloramphenicol, 25 $\mu\text{g ml}^{-1}$; and spectinomycin, 50 $\mu\text{g ml}^{-1}$.

P. alhagi LTYR-11Z has a 4.2-Mb genome. Construction of a mini-Tn5 transposon insertion mutant library of *P. alhagi* LTYR-11Z, plasmid rescue, and subsequent sequence analysis were carried out as described previously (74). Chromosomal gene deletion mutations in *P. alhagi* LTYR-11Z were generated via clustered regularly interspaced short palindromic repeats with Cas9 (CRISPR-Cas9) according to the method of Jiang et al. (75), with the spectinomycin resistance gene in the pTargetF plasmid replaced by a chloramphenicol resistance gene.

Screening for Tn5 transposon insertion mutants defective in attachment to wheat roots. Of 3,000 Tn insertion mutants in the library, 300 were randomly selected for screening of mutants defective in attachment to wheat roots. Seeds of winter wheat (*Triticum aestivum* L.) cultivar Zhengyin 1 were surface sterilized according to the protocol described previously (9). Surface-sterilized wheat seeds were placed in petri dishes containing 2 layers of damp sterile filter paper and germinated in the dark at 25°C for 2 days. The uniform-sized wheat seedlings were separately dipped in sterile saline solution (8.5 g NaCl liter⁻¹) containing bacterial suspensions of 10⁸ cells ml⁻¹ of the wild-type LTYR-11Z strain and Tn5 insertion mutants and incubated at 30°C and 50 rpm for 1 h. Approximately 0.05 g of wheat root was cut, washed twice gently in sterile saline to remove weakly bound bacteria, and then vortexed vigorously for 1 min in 1 ml of sterile saline. Then the bacterial suspension was used for serial dilutions that were plated on LB agar to determine the number of bacteria attached per gram of fresh wheat roots. The assays were repeated three times. Statistical analysis was performed using the Student *t* test, and a difference was considered statistically significant when *P* was ≤ 0.05 .

RNA extraction and transcriptome sequencing analysis. The wild-type LTYR-11Z strain and the Δ *cra* mutant were cultivated in LB medium under agitation at 30°C to an optical density at 600 nm (OD₆₀₀) of 1.0, and then bacterial cells were harvested and sent to BGI-Shenzhen for RNA extraction and transcriptome sequencing analysis. Briefly, three replicates of each culture were subjected to total RNA extraction and mRNA enrichment using the Ribo-Zero kit. Transcriptome sequencing was performed on the BGISEQ-500 platform as described previously (76). Quality-filtered reads were aligned to the reference genome sequence of *P. alhagi* LTYR-11Z (GenBank accession no. CP019706) using HISAT (77). The expression level of each gene was calculated using fragments per kilobase per million reads (FPKM). Student's *t* test was used to identify significantly up- or downregulated genes between the wild-type LTYR-11Z strain and the Δ *cra* mutant, and only those with a *P* value of < 0.05 and an absolute fold change of ≥ 1.5 were considered statistically significant.

qRT-PCR analysis. RNAs were extracted separately using the RNeasy Pure cell/bacteria kit (Qiagen Biotech Co., Ltd., Beijing, China) and then treated with the RNase-free DNase I to remove residual DNA according to the manufacturer's instructions. qRT-PCR was performed as described previously (78). The 16S rRNA gene was used as an internal control for normalization. The primer sets used for qRT-PCR analysis are shown in Table 3. Three biological replicates were used for each sample, and three technical replicates were performed for each biological replicate.

Construction of chromosomal *lacZ* fusion strains and β -galactosidase assays. The *lacZ* fusion reporter plasmids pK18mobsacB-*P_{eps}::lacZ*, pK18mobsacB-*P_{ydiV}::lacZ*, and pK18mobsacB-*P_{yedQ}::lacZ* were separately transformed into *E. coli* S17-1 λ pir and mated with *P. alhagi* strains according to the method described by Zhang et al. (79), and the chromosomal fusion reporter strains were selected by plating on LB plates containing 50 $\mu\text{g ml}^{-1}$ kanamycin and 25 $\mu\text{g ml}^{-1}$ chloramphenicol. The resulting fusion strains were grown to the stationary phase in LB broth or M9 salts (78) supplemented with the carbon source indicated (0.4%) and 0.5% Casamino Acids at 30°C, and then β -galactosidase activity was measured with *o*-nitrophenyl- β -galactoside (ONPG) as the substrate (80). The assays were carried out at least three times with individual samples in triplicate, and the Student *t* test was used to determine statistical significance.

Overexpression and purification of recombinant proteins. To express and purify His₆-tagged proteins His₆-Cra, His₆-YdiV, His₆-YedQ, and the His₆-FlhD₄C₂ complex, plasmids pET28a-*cra*, pET28a-*ydiV*, pET28a-*yedQ*, and pET28a-*flhDC* were transformed into *E. coli* BL21(DE3). *E. coli* strains carrying the recombinant plasmids were grown at 37°C in LB medium to an OD₆₀₀ of 0.5 and then induced with 0.5 mM isopropyl- β -D-1-thiogalactopyranoside (IPTG) for 8 h at 26°C. Bacterial cells were harvested and broken by sonication. Then the His-tagged proteins were purified using Ni-nitrilotriacetic acid (NTA) His-bind resin (Novagen, Madison, WI) according to the manufacturer's instructions. Purified recombinant proteins were dialyzed against phosphate-buffered saline (PBS) overnight at 4°C and then subjected to SDS-PAGE analysis to verify the purity. Protein concentrations were measured using the Bradford assay (81), and all proteins were stored at -80°C until use.

EMSAs. EMSAs were performed as described by Zhang et al. (79). Briefly, DNA probes with or without putative binding site mutations were amplified from defined regions of the promoters and then purified

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Description ^a	Reference or source
Strains		
<i>E. coli</i>		
BL21(DE3)	Host for expression vector pET28a	Novagen
BW20767	Conjugation donor strain harboring pRL27	74
S17-1 λ pir	λ pir lysogen of S17-1, <i>thi pro hsdR hsdM⁺ recA::RP4-2-Tc::Mu-Km::Tn7</i>	78
TG1	Host for cloning	Stratagene
<i>P. alhagi</i>		
WT	Wild-type <i>Pantoea alhagi</i> strain LTYR-11Z, Amp ^r	9
WT(pKT100)	Wild-type strain harboring plasmid PKT100	This study
WT(pBBR1MCS-1)	Wild-type strain harboring plasmid pBBR1MCS-1	This study
WT(pKT100-GFPmut3*)	Wild-type strain harboring expression construct pKT100-GFPmut3*	9
<i>cra::Tn</i> mutant	LTYR-11Z <i>cra::Tn</i> mutant isolate	This study
Δ <i>cra</i> mutant	<i>cra</i> deleted in LTYR-11Z	This study
Δ <i>cra</i> (PKT100) mutant	Δ <i>cra</i> mutant harboring plasmid PKT100	This study
Δ <i>cra</i> (pBBR1MCS-1) mutant	Δ <i>cra</i> mutant harboring plasmid pBBR1MCS-1	This study
Δ <i>cra</i> (pKT100- <i>cra</i>) mutant	Δ <i>cra</i> mutant harboring expression construct pKT100- <i>cra</i>	This study
Δ <i>cra</i> (pKT100-mCherry) mutant	Δ <i>cra</i> mutant harboring expression construct pKT100-mCherry	This study
Δ <i>cra</i> (pBBR1MCS1- <i>cra</i>) mutant	Δ <i>cra</i> mutant harboring expression construct pBBR1MCS1- <i>cra</i>	This study
Δ <i>cra</i> Δ <i>tuaG</i> mutant	<i>cra</i> and <i>tuaG</i> deleted in LTYR-11Z	This study
Δ <i>cra</i> Δ <i>tuaG</i> (pKT100- <i>cra</i>) mutant	Δ <i>cra</i> Δ <i>tuaG</i> mutant harboring expression construct pKT100- <i>cra</i>	This study
Δ <i>cra</i> Δ <i>tuaG</i> (pKT100- <i>tuaG</i>) mutant	Δ <i>cra</i> Δ <i>tuaG</i> mutant harboring expression construct pKT100- <i>tuaG</i>	This study
Δ <i>tuaG</i> mutant	<i>tuaG</i> deleted in LTYR-11Z	This study
Δ <i>tuaG</i> (pKT100- <i>tuaG</i>) mutant	Δ <i>tuaG</i> mutant harboring expression construct pKT100- <i>tuaG</i>	This study
Δ <i>ydiV</i> mutant	<i>ydiV</i> deleted in LTYR-11Z	This study
Δ <i>ydiV</i> (pKT100- <i>ydiV</i>) mutant	Δ <i>ydiV</i> mutant harboring expression construct pKT100- <i>ydiV</i>	This study
Δ <i>cra</i> Δ <i>ydiV</i> mutant	<i>cra</i> and <i>ydiV</i> deleted in LTYR-11Z	This study
Δ <i>cra</i> Δ <i>ydiV</i> (pKT100- <i>cra</i>) mutant	Δ <i>cra</i> Δ <i>ydiV</i> mutant harboring expression construct pKT100- <i>cra</i>	This study
Δ <i>cra</i> Δ <i>ydiV</i> (pKT100- <i>ydiV</i>) mutant	Δ <i>cra</i> Δ <i>ydiV</i> mutant harboring expression construct pKT100- <i>ydiV</i>	This study
Δ <i>yedQ</i> mutant	<i>yedQ</i> deleted in LTYR-11Z	This study
Δ <i>yedQ</i> (pKT100- <i>yedQ</i>) mutant	Δ <i>yedQ</i> mutant harboring expression construct pKT100- <i>yedQ</i>	This study
Δ <i>cra</i> Δ <i>yedQ</i> mutant	<i>cra</i> and <i>yedQ</i> deleted in LTYR-11Z	This study
Δ <i>pfkA</i> mutant	<i>pfkA</i> deleted in LTYR-11Z	This study
Plasmids		
pRL27	Km ^r , modified Tn5 plasmid	74
pKT100	Cloning vector, p15A replicon, Km ^r	78
pKT100- <i>cra</i>	<i>cra</i> in pKT100	This study
pKT100-mCherry	mCherry in pKT100	This study
pKT100-GFPmut3*	GFPmut3* in pKT100	9
pKT100- <i>yedQ</i>	<i>yedQ</i> in pKT100	This study
pKT100- <i>tuaG</i>	<i>tuaG</i> in pKT100	This study
pKT100- <i>ydiV</i>	<i>ydiV</i> in pKT100	This study
pBBR1MCS-1	Broad-host-range vector, <i>lacZ</i> α rep mob Cm ^r	84
pBBR1MCS1- <i>cra</i>	<i>cra</i> in pBBR1MCS-1	This study
pCas	CRISPR-Cas9 system plasmid used for in-frame deletion, Km ^r	75
pTargetF	CRISPR-Cas9 system plasmid used for in-frame deletion, Sp ^r	75
pTargetF1	pTargetF with spectinomycin resistance gene replaced by chloramphenicol resistance gene, Cm ^r	This study
pTargetT- Δ <i>cra</i>	Construct used for in-frame deletion of <i>cra</i>	This study
pTargetT- Δ <i>tuaG</i>	Construct used for in-frame deletion of <i>tuaG</i>	This study
pTargetT- Δ <i>ydiV</i>	Construct used for in-frame deletion of <i>ydiV</i>	This study
pTargetT- Δ <i>yedQ</i>	Construct used for in-frame deletion of <i>yedQ</i>	This study
pTargetT- Δ <i>pfkA</i>	Construct used for in-frame deletion of <i>pfkA</i>	This study
pK18mobsacB-P _{eps} :: <i>lacZ</i>	For <i>eps</i> promoter fusion to <i>P. alhagi</i> , Km ^r	This study
pK18mobsacB-P _{ydiV} :: <i>lacZ</i>	For <i>ydiV</i> promoter fusion to <i>P. alhagi</i> , Km ^r	This study
pK18mobsacB-P _{yedQ} :: <i>lacZ</i>	For <i>yedQ</i> promoter fusion to <i>P. alhagi</i> , Km ^r	This study
P _{cdrA} - <i>lacZ</i>	<i>cdrA</i> promoter of <i>P. aeruginosa</i> PAK fused to promoterless <i>lacZ</i> on pDN19lacZ Ω , Sp ^r Tc ^r	32
pET28a	Expression vector with N-terminal hexahistidine affinity tag, Km ^r	Novagen
pET28a- <i>cra</i>	<i>cra</i> in pET28a	This study
pET28a- <i>ydiV</i>	<i>ydiV</i> in pET28a	This study
pET28a- <i>flhDC</i>	<i>flhDC</i> in pET28a	This study
pET28a- <i>yedQ</i>	<i>yedQ</i> in pET28a	This study

^aCm^r, Km^r, Tc^r, Sp^r, and Amp^r represent resistance to chloramphenicol, kanamycin, tetracycline, spectinomycin, and ampicillin at 25, 50, 20, 50, and 100 μ g ml⁻¹, respectively.

TABLE 3 Primers used in this study

Primer	5'→3' sequence ^a	Purpose
Cat-F	CCCTCGAGCTGAACAGGAGGGACAGC	To generate pTargetF1
Cat-R	AAAAGTACTCGGTAAACCAGCAATAGACAT	To generate pTargetF1
<i>cra</i> -sg20-F-SpeI	GTCCTAGGTATAATACTAGTAGCATCCTTTCTACCAGCGCGTTTTAGAGCTAGAAATAGC	To generate sgRNA with an N ₂₀ sequence for targeting <i>cra</i> locus
<i>ydiV</i> -sg20-F-SpeI	GTCCTAGGTATAATACTAGTAAATTAACCCTGACTAATTTGTTTTAGAGCTAGAAATAGC	To generate sgRNA with an N ₂₀ sequence for targeting <i>ydiV</i> locus
<i>yedQ</i> -sg20-F-SpeI	GTCCTAGGTATAATACTAGTGAATCAACACTGTTTGATGTTTTAGAGCTAGAAATAGC	To generate sgRNA with an N ₂₀ sequence for targeting <i>yedQ</i> locus
<i>tuaG</i> -sg20-F-SpeI	GTCCTAGGTATAATACTAGTTAAAAACCGCGTTTGCATGAGTTTTAGAGCTAGAAATAGC	To generate sgRNA with an N ₂₀ sequence for targeting <i>tuaG</i> locus
<i>pfkA</i> -sg20-F-SpeI	GTCCTAGGTATAATACTAGTTATTTTACAGCGCTGGAAAGTTTTAGAGCTAGAAATAGC	To generate sgRNA with an N ₂₀ sequence for targeting <i>pfkA</i> locus
sg20-R	CTCAAAAAAGCACCGACTCGG	To generate sgRNA
<i>cra</i> -up-F	GTTGTTGTGCCGAGGAT CTGGATGCGTTTCTAGTACGT	To generate pTargetT- <i>Acra</i>
<i>cra</i> -up-R	TGACATAGCTGGCGGTGGT	To generate pTargetT- <i>Acra</i>
<i>cra</i> -down-F	ACCACCGCCAGCTATGTCA TTAGGCGCAATCTGGTTCCG	To generate pTargetT- <i>Acra</i>
<i>cra</i> -down-R-Sall	GCGTCGACTGCAACATCTGAAAAATAAAC	To generate pTargetT- <i>Acra</i>
<i>cra</i> -CF-Sall	GCGTCGACGTGAACTGGATGAAATAGCG	To generate pBBR1MCS1- <i>cra</i>
<i>cra</i> -CR-BamHI	CGGGATCCTTACGTACGACTTAATTTCCGC	To generate pBBR1MCS1- <i>cra</i>
<i>cra</i> -CR-F-BamHI	CGGGATCCGTGAACTGGATGAAATAGCG	To generate pKT100- <i>cra</i>
<i>cra</i> -CR-R-XbaI	GCTCTAGATTACGTACGACTTAATTTCCGC	To generate pKT100- <i>cra</i>
<i>cra</i> -PF-BamHI	CGGGATCCGTGAACTGGATGAAATAGCG	To generate pET28a- <i>cra</i>
<i>cra</i> -PR-EcoRI	CGGAATTCTTACGTACGACTTAATTTCCGC	To generate pET28a- <i>cra</i>
<i>ydiV</i> -up-F	CCGAGTCGGTGCTTTTTTTGAG CCAAGAAACATAAGCAGCCATAA	To generate pTargetT- <i>YdiV</i>
<i>ydiV</i> -up-R	ATCCTGCGGCACAACAAC	To generate pTargetT- <i>YdiV</i>
<i>ydiV</i> -down-F	GTTGTTGTGCCGAGGAT TCAGGTAAGCGATCACTGCC	To generate pTargetT- <i>YdiV</i>
<i>ydiV</i> -down-R-sall	GCGTCGACCTGTTTCAGCGGTGTTGG	To generate pTargetT- <i>YdiV</i>
<i>ydiV</i> -CF-BamHI	CGGGATCCATGACAACAACAGTTTTGCTGA	To generate pKT100- <i>ydiV</i>
<i>ydiV</i> -CR-EcoRI	CGGAATTCTCAGCATTGACCGTCAGAAA	To generate pKT100- <i>ydiV</i>
<i>ydiV</i> -PF-BamHI	CGGGATCCATGACAACAACAGTTTTGCTGA	To generate pET28a- <i>ydiV</i>
<i>ydiV</i> -PR-EcoRI	CGGAATTCTCAGCATTGACCGTCAGAAA	To generate pET28a- <i>ydiV</i>
<i>flhDC</i> -PF-BamHI	CGGGATCCATGGGTACATCCGAATTATTA	To generate pET28a- <i>flhDC</i>
<i>flhDC</i> -PR-EcoRI	CGGAATTCTTAAACGGCGTGTTAACCT	To generate pET28a- <i>flhDC</i>
<i>yedQ</i> -up-F	CCGAGTCGGTGCTTTTTTTGAG CCTGCCGTATCTGTTGA	To generate pTargetT- <i>YedQ</i>
<i>yedQ</i> -up-R	TCAGCCATACCGAGACCA	To generate pTargetT- <i>YedQ</i>
<i>yedQ</i> -down-F	TGGTCTCGGTATGGCTGG TACACGGTAAGTATCGGCATTG	To generate pTargetT- <i>YedQ</i>
<i>yedQ</i> -down-R-sall	GCGTCGACTTGCAGGTAATGTTTGATGG	To generate pTargetT- <i>YedQ</i>
<i>yedQ</i> -CF-BamHI	CGGGATCCATGGCACTTGATGTCTACACACT	To generate pKT100- <i>yedQ</i>
<i>yedQ</i> -CR-EcoRI	CGGAATTCTTAAAGGTTAAGGTTACACAGC	To generate pKT100- <i>yedQ</i>
<i>tuaG</i> -up-F	CCGAGTCGGTGCTTTTTTTGAG GATTCTGGCGGTGACCGA	To generate pTargetT- <i>ΔtuaG</i>
<i>tuaG</i> -up-R	CGATGTTACGCCAGGTTTG	To generate pTargetT- <i>ΔtuaG</i>
<i>tuaG</i> -down-F	CAAACCTGGCGTAACATCG AAATCGAAAGGCGTGGCT	To generate pTargetT- <i>ΔtuaG</i>
<i>tuaG</i> -down-R-Sall	GCGTCGACAGCTGTGCTGCCGTAAGA	To generate pTargetT- <i>ΔtuaG</i>
<i>tuaG</i> -CF-BamHI	CGGGATCCGTGCCAGCCGTTCCGCG	To generate pKT100- <i>tuaG</i>
<i>tuaG</i> -CR-EcoRI	CGGAATTCTCACTCCCGGAACAAAACGTT	To generate pKT100- <i>tuaG</i>
<i>pfkA</i> -up-F	CCGAGTCGGTGCTTTTTTTGAG AACAGTTTCTGTTCCG	To generate pTargetT- <i>ΔpfkA</i>
<i>pfkA</i> -up-R	CCATCATAAATGCCACAACT	To generate pTargetT- <i>ΔpfkA</i>
<i>pfkA</i> -down-F	AGTTTGTGGCATTATGATGG CGCTTATGCGATTGAACGT	To generate pTargetT- <i>ΔpfkA</i>
<i>pfkA</i> -down-R-Sall	ACGCGTCGACCCGGTTTCTGCTGTGTAG	To generate pTargetT- <i>ΔpfkA</i>
<i>P_{eps}</i> -F-BamHI	CGGGATCCGGGCGGTAGCGTGCTTAA	pK18mobsacB- <i>P_{eps}::lacZ</i>
<i>P_{eps}</i> -R-XbaI	GCTCTAGACCAACTTTAGTTTCGTTTCATC	pK18mobsacB- <i>P_{ydiV}::lacZ</i>
<i>P_{ydiV}</i> -F-BamHI	CGGGATCCCTCCATGCCTCTACCAGCAA	pK18mobsacB- <i>P_{ydiV}::lacZ</i>
<i>P_{ydiV}</i> -R-XbaI	GCTCTAGATCAGCAAAAAGTGTGTTGTCAT	pK18mobsacB- <i>P_{eps}::lacZ</i>
<i>P_{yedQ}</i> -F-BamHI	CGGGATCCCGATGAAGTCGTGTTTGC	pK18mobsacB- <i>P_{yedQ}::lacZ</i>
<i>P_{yedQ}</i> -R-XbaI	GCTCTAGAAGTGTGTAGACATCAAGTCCAT	pK18mobsacB- <i>P_{yedQ}::lacZ</i>
<i>eps</i> -EMSA-F	ATTTAACAGCATTGGTAGCTGAA	EMSA
<i>eps</i> -EMSA-R	TGAATTATCATCACTATTGCTGA	EMSA
<i>eps</i> -EMSA-M-F	ATTTAACAGCATTGGTACACAGGCGCCAGGGCG	To generate mutated <i>P_{eps}</i> fragment for EMSA
<i>ydiV</i> -EMSA-F	TCCATGCCTCTACCAGCAA	EMSA
<i>ydiV</i> -EMSA-R	TTTTTTTAGCCATAAAAAAGTGG	EMSA
<i>ydiV</i> -F1-EMSA-R	AAGATTTAGTGATCATTCTCGTTGTTTCCGG	To generate fragment 1 of <i>P_{ydiV}</i> for EMSA
<i>ydiV</i> -F2-EMSA-F	TAAATCTTATTTATCCTGGCTGA	To generate fragment 2 of <i>P_{ydiV}</i> for EMSA
<i>ydiV</i> -F2-EMSA-M-F	TAAATCTTATTTATCCTGTACATTCAAGCCCCAA	To generate mutated fragment 2 of <i>P_{ydiV}</i> for EMSA
<i>yedQ</i> -EMSA-F	CGATGAAGTCGTGTTTGC	EMSA

(Continued on next page)

TABLE 3 (Continued)

Primer	5'→3' sequence ^a	Purpose
yedQ-EMSA-R	TATAAAAAACCGCAGCAAAATAT	EMSA
yedQ-M1-F	TGTCCCCGATATCGGCGGCGTC	To generate mutated P_{yedQ} fragment for EMSA
yedQ-M1-R	CGGCGAATCTCCCTGTGCAGCATGCCTGAAAG	To generate mutated P_{yedQ} fragment for EMSA
yedQ-M2-F	TTTCAGGCATGCTGCACAGGGAGATTCGCCG	To generate mutated P_{yedQ} fragment for EMSA
yedQ-M2-R	AAAGCGCTTCCCTATAAAAAACCGCAGCAAAATATCATGACTCGC	To generate mutated P_{yedQ} fragment for EMSA
flhA-EMSA-F	GACTGTTTATTAGCGGACGATT	EMSA
flhA-EMSA-R	GACTACCTGCGTTAGTTACGTT	EMSA
16S-RT-F	CGAGCGCAACCCCTATCC	qRT-PCR
16S-RT-R	CGGACTACGACGCACTTTATG	qRT-PCR
wza-RT-F	GGCGTGTTTGTATTTCGTC	qRT-PCR
wza-RT-R	CAGCGGGTAAGCGGTGTT	qRT-PCR
tuaG-RT-F	TGCACCTGCGACGGAATA	qRT-PCR
tuaG-RT-R	AGGGCGAAATCCAGTCC	qRT-PCR
ydiV-RT-F	GCAGGCGCTGGAATTAGG	qRT-PCR
ydiV-RT-R	CAGCTGATACGATGAAACAAA	qRT-PCR
yedQ-RT-F	GTTGCATTGATTATTATGCTGGTC	qRT-PCR
yedQ-RT-R	TCGCATTGTGAGCGGAAG	qRT-PCR

^aUnderlined sites indicate restriction enzyme cutting sites added for cloning. Letters in boldface denote the annealing regions for overlap PCR. Mutations introduced in the putative Cra binding sites are shaded gray.

and quantified. EMSAs were performed by adding increasing amounts of purified recombinant protein to DNA probe (2 ng μl^{-1}) in EMSA buffer (20 mM Tris-HCl [pH 7.4], 4 mM MgCl_2 , 100 mM NaCl, 1 mM dithiothreitol, 10% glycerol). In relevant experiments, various concentrations of metabolites or His₆-YdiV were added to the indicated reaction mixtures. The binding reaction mixtures were incubated for 30 min at room temperature and then loaded onto a native 6% polyacrylamide gel containing 5% glycerol in 0.5× Tris-borate-EDTA (TBE) buffer. After electrophoresis at 4°C for 2 to 3 h at 100 V, gels were stained with SYBR green and imaged using the Universal Hood II system (Bio-Rad Laboratories, Inc.).

EPS quantification. EPS quantification was performed as described by Santaella et al. (82) with minor modifications. Briefly, the *P. alhagi* wild-type and mutant strains were grown in 100 ml of LB medium at 30°C with shaking at 180 rpm. After 3 days of cultivation, the stationary-phase cultures were centrifuged at 6,000 rpm for 15 min at 4°C and bacterial cells were removed. Ice-cold ethanol (2.5 volumes) was added to the supernatants containing soluble EPS, and the mixtures were left overnight at 4°C under agitation. Then the EPS precipitated was harvested by 20 min of centrifugation at 10,000 rpm at 4°C and resuspended in 1 ml of sterile deionized water. The EPS solutions were frozen at −80°C for 24 h and then dried by lyophilization for 48 h. The dry weight of EPS was quantified and expressed as milligrams per liter of culture.

Biofilm formation assay. Biofilm formation was measured as described previously (56). Briefly, overnight bacterial cultures were diluted to an OD₆₀₀ of 0.025 in fresh LB medium and inoculated into each well of a 96-well microtiter plate. After incubation at 30°C for 48 h, culture supernatant was removed and the wells were washed gently with distilled water two times. Biofilms were stained with 0.1% (wt/vol) crystal violet for 15 min, followed by two washes with distilled water. Ethanol (200 μl) was added to each well, the wells were incubated at room temperature for 10 min, and then the absorbance of the eluted solution was read at 590 nm. The assays were performed three times, with samples assayed in octuplicate.

Swimming motility assay. Swimming motility was evaluated in 0.3% agar tryptone plates (1% tryptone, 0.5% NaCl, 0.3% Bacto agar [Difco]) as described previously (83). Briefly, bacterial strains were grown overnight at 30°C in LB broth and then diluted to an OD₆₀₀ of 0.1 in fresh LB broth. Two microliters of bacterial suspension was injected into the center of swimming motility plates. After the plates were incubated at 22°C for 10 h, motility halos were measured. Assays were repeated at least three times independently.

Measurement of DGC activity *in vitro*. The DGC activity of YedQ was determined using an *in vitro* enzyme activity assay as described by Nesbitt et al. (57), with minor modifications. Briefly, the reaction mixture contained 50 mM Tris-HCl (pH 7.5), 5 mM MgCl_2 , and 100 μM GTP in a 200- μl volume. The enzymatic reactions were initiated by adding 3.5 μM YedQ protein, and reaction mixtures were immediately incubated at 30°C. Reactions were terminated at various times by heating the samples at 95°C for 5 min. Denatured protein was removed from the reaction mixture through centrifugation, and then the supernatant was filtered through a 0.22- μm -pore membrane, followed by reverse-phase high-performance liquid chromatography (HPLC) analysis using a C₁₈ Waters Symmetry 5- μm column (4.6 by 250 mm). The mobile phase consisted of 0.1% formic acid water (A) and 0.1% formic acid acetonitrile (B), with a linear gradient from 5% to 50% B over 15 min at a flow rate of 0.3 ml min^{−1}. The detection wavelength was 270 nm. Authentic GTP and c-di-GMP (Sigma) were used as standards to determine retention times of the nucleotides.

Measurement of the intracellular c-di-GMP levels. Quantification of intracellular c-di-GMP was performed as described previously (32). The plasmid P_{cdrA} -*lacZ* containing the *cdrA* promoter of *Pseudomonas aeruginosa* PAK fused to promoterless *lacZ* was transformed into *E. coli* S17-1 λ pir and then mated with *P. alhagi* strains as described above. The intracellular levels of c-di-GMP were assessed by measuring the β -galactosidase activity, and data are presented as fold changes over the wild-type levels.

Plant assays. After germination, 2-day-old wheat seedlings were inoculated with bacterial suspensions of 10^8 cells ml^{-1} in 1/2 MS medium (9) without a carbon source for 1 h at 30°C and 50 rpm. Attachment assays were performed as described above. For endophytic colonization assays, each inoculated seedling was transferred to a hydroponic system composed of 3 ml of the 1/2 MS medium without a carbon source in a glass tube and grown at 25°C with a photoperiod of 14 h/10 h light/dark. One, 4, 7, and 10 days after inoculation, the wheat roots were cut, weighed, and surface sterilized according to the procedure described by Balsanelli et al. (14). The surface-sterilized roots were then homogenized, serially diluted, and plated on LB agar to determine the number of endophytic bacteria colonizing internal root tissues.

Competition assays were also performed as described above, but using a mixed bacterial suspension containing wild-type LTYR-11Z carrying plasmid pKT100 and the *cra* mutant carrying plasmid pBBR1MCS-1 at 1:1. The total number of bacteria attached to and internalized by wheat roots was determined as described before, while antibiotic resistance was used to distinguish the strains. Additionally, wild-type LTYR-11Z carrying pBBR1MCS-1 and the *cra* mutant carrying pKT100 were used for competition assays.

The wheat seedlings were used to collect root exudates. Briefly, 4-day-old wheat seedlings germinated in petri dishes were transferred to a hydroponic system with 10 seedlings per 250-ml flask containing 50 ml 1/2 MS liquid medium without a carbon source. After 7 and 14 days of incubation at 25°C in a plant growth chamber (14 h light/10 h dark), plants were removed and the root exudates were collected. Then root exudates were filter sterilized through a 0.22- μm -pore membrane and stored at -20°C until use. Bacterial strains were grown in LB medium to an OD_{600} of 1.0, washed twice in fresh 1/2 MS medium, and resuspended in wheat root exudates or sterile 1/2 MS medium to give a concentration of 10^8 cells ml^{-1} . After incubation for 9 h at 30°C in the dark with gentle shaking at 100 rpm, cell densities and β -galactosidase activities were determined.

To investigate whether carbon sources play a role in the attachment of wheat roots by *P. alhagi* LTYR-11Z, 2-day-old wheat seedlings were inoculated with bacterial suspensions of 10^8 cells ml^{-1} in 1/2 MS medium supplemented with 0.4% glucose or pyruvate as the sole carbon source. After 1 and 15 h of incubation at 30°C and 50 rpm, the number of attached cells of each strain was determined as described above. Bacterial suspensions incubated for the same time in 1/2 MS medium with the carbon source indicated but without transplanted wheat seedlings were used as a control to exclude the possibility that the difference in attachment capacity was due to the different growth rates in the presence of glucose versus pyruvate. In addition, wheat seedlings were inoculated with a mixture of the GFP-labeled wild-type LTYR-11Z and the mCherry-labeled *cra* mutant at 1:1 in 1/2 MS medium supplemented with 0.4% pyruvate and incubated at 30°C and 50 rpm for 15 h. After incubation, wheat roots were gently washed to remove weakly bound bacteria and then stained with 4',6-diamidino-2-phenylindole (DAPI [300 nM in PBS]) for 1 min before being observed using a laser-scanning confocal microscope (Leica, Germany). The excitation/emission wavelengths were 358/461 (DAPI), 488/520 (GFP), and 561/595 nm (mCherry) nm, respectively.

Accession number(s). The RNA-seq data from this study have been submitted to the Sequence Read Archive database under accession no. [SRP136873](https://doi.org/10.1101/36873).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.00054-18>.

SUPPLEMENTAL FILE 1, XLS file, 0.2 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.2 MB.

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