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RESEARCH ARTICLE
Host-Microbe Biology

Diverse Bacterial Genes Modulate Plant Root Association by Beneficial Bacteria

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ABSTRACT The plant rhizosphere harbors a diverse population of microorganisms, including beneficial plant growth-promoting bacteria (PGPB), that colonize plant roots and enhance growth and productivity. In order to specifically define bacterial traits that contribute to this beneficial interaction, we used high-throughput transposon mutagenesis sequencing (TnSeq) in two model root-bacterium systems associated with *Setaria viridis*: *Azoarcus olearius* DQS4^T and *Herbaspirillum seropedicae* SmR1. This approach identified ~100 significant genes for each bacterium that appeared to confer a competitive advantage for root colonization. Most of the genes identified specifically in *A. olearius* encoded metabolism functions, whereas genes identified in *H. seropedicae* were motility related, suggesting that each strain requires unique functions for competitive root colonization. Genes were experimentally validated by site-directed mutagenesis, followed by inoculation of the mutated bacteria onto *S. viridis* roots individually, as well as in competition with the wild-type strain. The results identify key bacterial functions involved in iron uptake, polyhydroxybutyrate metabolism, and regulation of aromatic metabolism as important for root colonization. The hope is that by improving our understanding of the molecular mechanisms used by PGPB to colonize plants, we can increase the adoption of these bacteria in agriculture to improve the sustainability of modern cropping systems.

IMPORTANCE There is growing interest in the use of associative, plant growth-promoting bacteria (PGPB) as biofertilizers to serve as a sustainable alternative for agriculture application. While a variety of mechanisms have been proposed to explain bacterial plant growth promotion, the molecular details of this process remain unclear. The current research supports the idea that PGPB use in agriculture will be promoted by gaining more knowledge as to how these bacteria colonize plants, promote growth, and do so consistently. Specifically, the research seeks to identify those bacterial genes involved in the ability of two, PGPB strains, *Azoarcus olearius* and *Herbaspirillum seropedicae*, to colonize the roots of the C4 model grass *Setaria viridis*. Applying a transposon mutagenesis (TnSeq) approach, we assigned phenotypes and function to genes that affect bacterial competitiveness during root colonization. The results suggest that each bacterial strain requires unique functions for root colonization but also suggests that a few, critical functions are needed by both bacteria, pointing to some common mechanisms. The hope is that such information can be exploited to improve the use and performance of PGPB in agriculture.

KEYWORDS bacterium-root colonization, beneficial bacteria, gene functionality, transposon mutagenesis

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Plant health and development are influenced by a broad range of microorganisms inhabiting the root rhizosphere, including beneficial associative bacteria. These bacteria are usually minor components of the rhizosphere microbial community but have been shown to significantly enhance plant growth and yield (1, 2). This ability to enhance plant growth has been attributed to diverse mechanisms, including biological nitrogen fixation, production of phytohormones, enhancement of nutrient uptake (siderophore and phosphate solubilization), and biocontrol of pathogens and pests (3). Plant growth-promoting bacteria (PGPB) can colonize roots either on the root surface or as endophytes without eliciting a noticeable plant defense response (4, 5). Unlike some other bacterium-plant interactions that show strict host specificity, many PGPB can colonize a wide variety of plant species, including agriculturally important members of the *Poaceae* family, such as rice, maize, wheat, and a variety of bioenergy grasses (6, 7). PGPB strains have been described for several host plants, including *Setaria viridis*, a model C_4 plant, which is a close relative to a variety of bioenergy grasses. In a previous study, we demonstrated that *S. viridis* under lab conditions can obtain up to 100% of its nitrogen needs through biological nitrogen fixation mediated by diazotrophic PGPB (8). Among the strains used were the betaproteobacteria *Azoarcus olearius* and *Herbaspirillum seropedicae*, which exhibit strong growth-promoting ability (4, 9–12). *Azoarcus olearius* DQS4^T was originally isolated from oil-contaminated soil (13) and, based on its genome sequence, this strain shows high similarity with the well-studied *A. olearius* strain BH72 (14). Strain DQS4^T can colonize the roots of rice and *Setaria* to high levels and increase below- and above-ground biomass (9). *H. seropedicae* SmR1 is a well-studied endophytic bacterium that colonizes several plants, including maize, wheat, and *Setaria*. Recently, *in situ* metabolomic profiling of *S. viridis* roots colonized by SmR1 demonstrated that inoculation induced a wide variety of plant metabolic changes, including those affecting nitrogen and phytohormone levels (15). Thus, similar to many other, better-studied plant-microbe associations, PGPB inoculation appears to profoundly affect the metabolism of its host.

To identify specific PGPB genes that contribute to plant root colonization, we applied transposon mutagenesis sequencing (TnSeq) coupled with random barcoding (RB-TnSeq). This technique assesses gene functionality in mutant strains through the generation of reusable libraries of unique and mapped mutant insertions (16, 17). TnSeq is a powerful and sensitive approach to identify bacterial gene functions that play roles in bacterial fitness under researcher-defined growth conditions (16–18). Starting with information on the location and frequency of each mutation in the population, the change in mutational frequency after passage of the population through an environmental challenge (e.g., plant root colonization) leads to the identification of genes that are either essential, important, or detrimental to growth under that condition. For example, TnSeq of *Streptococcus pneumoniae* identified genes essential for bacterial basal growth, as well as genes involved in transcriptional regulation and carbohydrate transport (17). In the PGPB *Pseudomonas simiae*, TnSeq revealed genes involved in carbon metabolism and motility that could enhance or suppress colonization of *Arabidopsis* roots (19). In the present work, we applied standard TnSeq and a variant of TnSeq that uses random DNA barcodes to measure strain abundance (RB-TnSeq) to construct mutant libraries of strains DQS4^T and SmR1, respectively, and to then evaluate genetic contributions to fitness on the host plant *Setaria viridis*.

RESULTS

Overview of TnSeq analysis. Transposon sequencing is a high-throughput tool used to generate a large bacterial mutant population. Gene essentiality is defined as its importance in maintaining competitive fitness under a condition of interest (Fig. 1A).

For this study, two PGPB bacterial species were used, *Azoarcus olearius* DQS4^T and *Herbaspirillum seropedicae* SmR1, both diazotrophic and capable of promoting plant growth (8, 9, 11–13, 20). The mutagenized pool of each strain was inoculated onto *Setaria viridis* roots or a carbon augmented soil control with no plants. Root-associated

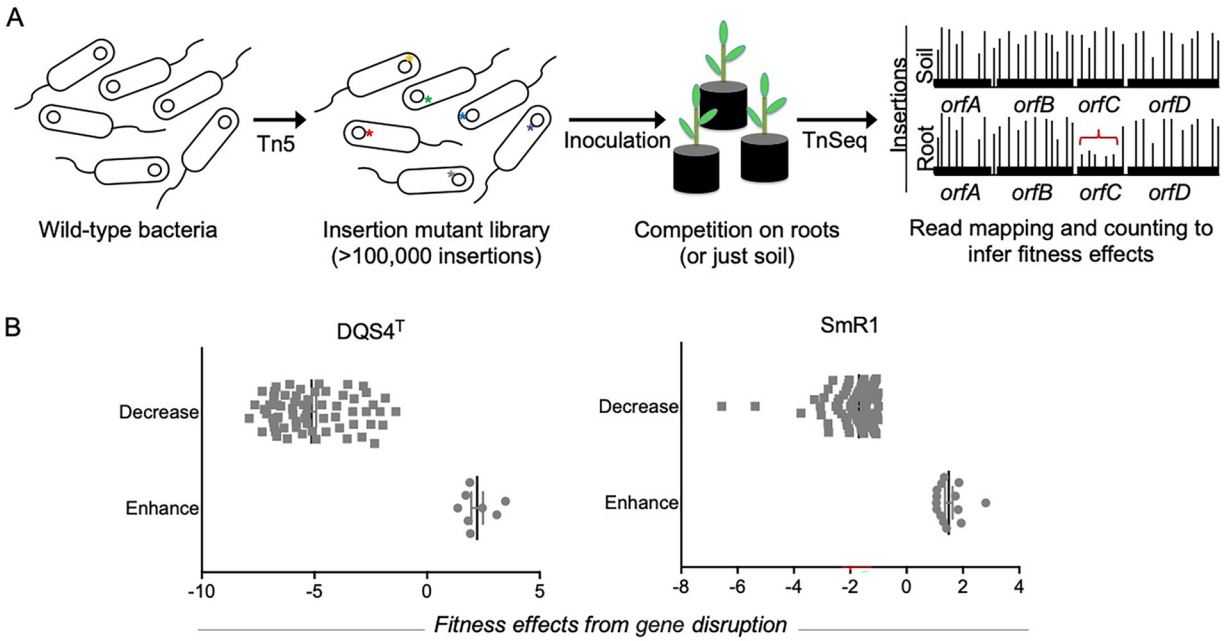

FIG 1 TnSeq representation and determination. (A) Insertion mutant library generated by a mariner transposon system from wild-type bacteria. A mutant pool of each bacterium (DQS4^T or SmR1) was inoculated onto *Setaria viridis*. Surviving mutant strains were recovered from the roots or soil without plants, at 10 days after inoculation, and the abundance of each insertion was quantified by TnSeq. (B) Categorization based on gene essentiality. Genes with low insertion counts within the root population generating negative fitness scores (<-1) were categorized as decreased root colonization (shown as square in both graphs). Genes with overrepresented insertion counts were given positive fitness scores (>1) and categorized as enhanced root colonization (shown as dots in both graphs). Plots show fitness scores obtained from the average values of root samples across biological replicates ($n = 4$).

bacterial cells were recovered, transposon DNA was sequenced, and resulting reads were mapped to each bacterial genome. The relative incidence of a specific mutation (or lack thereof) was then determined and used to calculate fitness values. By high-throughput sequence analysis of insertion mutants, we covered about 3,692 genes of DQS4^T and 3,878 genes of SmR1 distributed throughout either strain's genome. Thus, regardless of method, very good coverage of each genome was achieved. In the case of DQS4^T transposon mutagenesis, 89.2% of 4,135 total genes were mapped to a gene harboring at least 1 insertion event. For the SmR1 library, 81.1% of the 4781 genes had identified mutations. We assume that genes without an insertion in the library likely identify those essential for bacterial growth in culture.

Our screening identified 89 and 130 gene mutations that significantly affected the ability of strains DQS4^T and SmR1 to colonize *S. viridis* roots, respectively. Given that our interest was to identify genes that exclusively affected root colonization, we excluded from further consideration any genes that also affected fitness under soil conditions (see Fig. S1a). The genes affecting root colonization were categorized based on the phenotypes as either (i) enhanced fitness value, mutations that increased root association (fitness score ≥ 1); or (ii) decreased fitness value, mutations that impaired root association (fitness score ≤ -1) (Fig. 1B; see also Fig. S1b in the supplemental material). For the complete list of gene mutations affecting fitness under each condition, see Table S2.

Unsurprisingly, many of the genes identified lacked a clear functional annotation, especially for *A. olearius* DQS4^T. Even though each strain showed a distinct pattern of mutations that affected fitness, a few common gene functions (e.g., those involved in chemotaxis and cell wall recycling) were identified impairing root colonization in both strains. The most common COG categories important for SmR1 root colonization were those involved in amino acid transport and metabolism, followed by energy production and conversion and coenzyme transport and metabolism. In the case of DQS4^T

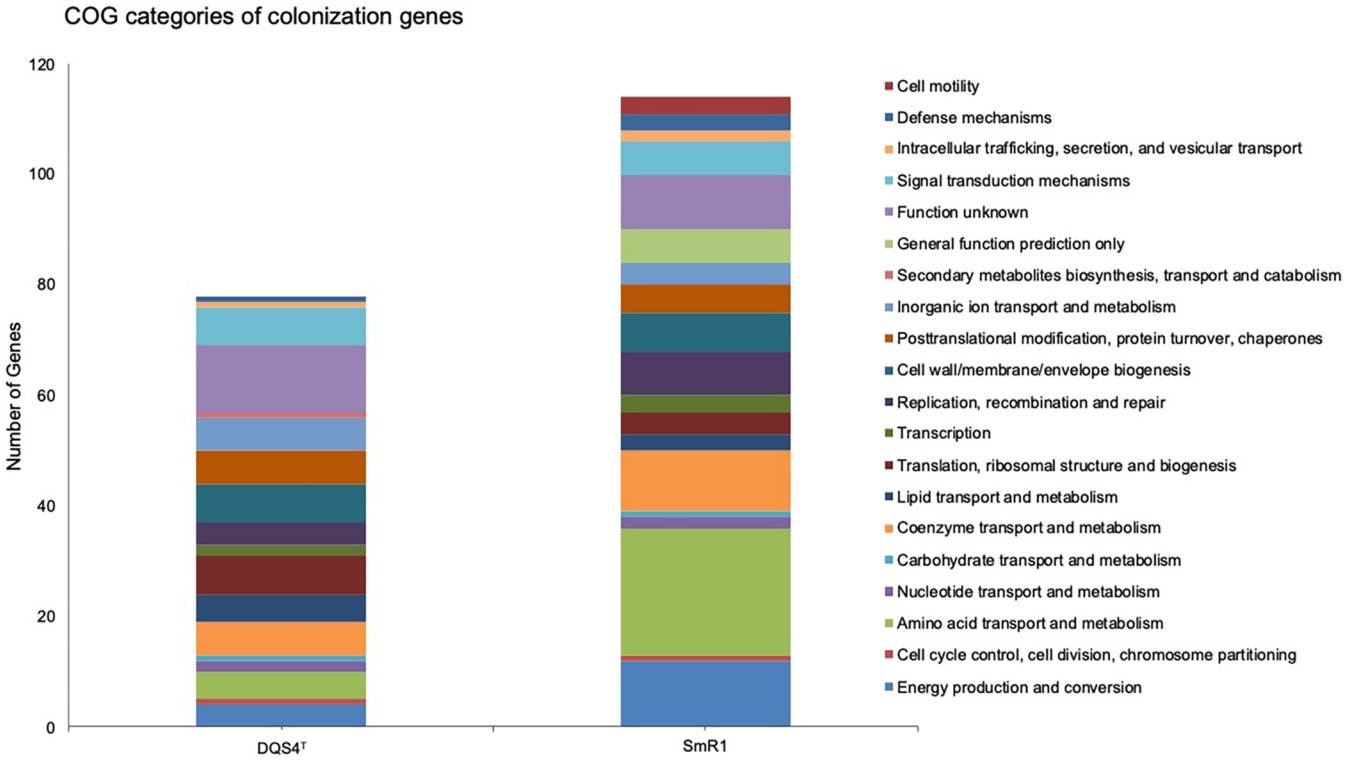

FIG 2 Cluster of orthologous group (COG) categories of colonization genes. The distributions of genes significantly increasing or decreasing root colonization when mutated are shown. The color legend of dominant COG categories is shown separately for *Azoarcus olearius* DQS4^T and *Herbaspirillum seropedicae* SmR1.

the most common COGs were cell wall/membrane/envelope biogenesis and signal transduction, followed by translation, ribosomal structure and biogenesis, and inorganic ion transport and metabolism (Fig. 2). Although we cannot rule out that the different mutagenesis methods could contribute to the different fitness profiles, the overall mutational coverage of the genomes of both strains suggest that such effects are likely minor. Hence, the results seem to suggest that any given PGPB strain will have different major requirements for root colonization reflecting the specific metabolic needs of the bacterium.

Transposon mutations that benefit bacterial root association. Gene mutations that positively impacted the ability of bacterial strains to colonize roots were defined as those with a fitness score ≥ 1. We identified 8 and 14 genes in this category in *A. olearius* and *H. seropedicae*, respectively (Table 1). This list included the *Azoarcus* gene predicted to encode a pyrroloquinoline quinone (PQQ)-dependent alcohol dehydrogenase (ADH; DQS_RS19730), which is involved in the oxidoreductase process of ADH. Based on sequence alignment with *Azoarcus olearius* BH72, the DQS_RS19730 gene encodes an ExaA5 protein that was previously reported to be induced upon exposure to ethanol as a carbon source (21). In our study, we found that *exaA5* plays an important role in root colonization. Disruption of this gene enhanced root colonization of *S. viridis* roots (Fig. 3A). However, the mutant was less competitive in root colonization than the wild type at 1 and 3 days after inoculation but seemed to recover to wild-type levels by 5 days after inoculation (d.a.i.) (Fig. 3B). An insertion mutation in the gene encoding a diguanylate cyclase (DQS_RS13665) significantly increased bacterial root colonization 3 and 5 days postinoculation (Fig. 3A) and showed the same trend in competition with the wild type (Fig. 3B). Diguanylate cyclases (DGCs) with a GGDEF active-site motif produce cyclic di-GMP (c-di-GMP) and play a major role in the transition between motile and sessile bacterial lifestyles (22–27). We also identified an iron regulator outer membrane protein, TonB (DQS_RS15430), presumably required for Fe^{3+}

TABLE 1 Mutations that enhanced DQS4^T or SmR1 root fitness colonization of *S. viridis*

Strain[a]	Locus ID	Gene annotation	Fitness score
DQS4^T	DQS_RS19730	PQQ-binding-like beta-propeller repeat protein ExaA5	1.36
	DQS_RS15440	Peptidase	2.45
	DQS_RS15145	Sigma-54-dependent Fis family transcriptional regulator	1.89
	DQS_RS13665	Histidine kinase sensor domain-containing diguanylate cyclase	1.92
	DQS_RS15430	TonB-dependent receptor	1.72
	DQS_RS08695	Ribonuclease III	3.09
	DQS_RS16645	DUF502 domain-containing protein	1.82
	DQS_RS15445	TonB-dependent siderophore receptor	3.47
SmR1	HSERO_RS14975	Flagellar motor protein MotA	1.05
	HSERO_RS10140	Flagellar biosynthesis protein FlhB	1.06
	HSERO_RS10305	Flagellar motor switch protein FliG	1.06
	HSERO_RS06285	Methyl-accepting chemotaxis protein	1.07
	HSERO_RS02815	HxlR family transcriptional regulator	1.20
	HSERO_RS10150	Flagellar biosynthesis regulator FlhF	1.24
	HSERO_RS10255	Flagellar biosynthesis protein FliQ	1.31
	HSERO_RS10310	Flagellar M-ring protein FliF	1.33
	HSERO_RS23885	XRE family transcriptional regulator	1.41
	HSERO_RS13885	Histidine kinase	1.73
	HSERO_RS14985	Transcriptional regulator	1.83
	HSERO_RS13890	LuxR family transcriptional regulator	1.85
	HSERO_RS20835	ABC transporter substrate-binding protein	1.93
	HSERO_RS08080	Poly[D(−)-3-hydroxyalkanoate] depolymerase	2.80

[a]DQS4^T, *Azoarcus olearious*; SmR1, *Herbaspirillum seropedicae*.

uptake (Fig. 3C). This protein was reported to interact with ExbA and ExbB, forming a complex that regulates iron acquisition (28).

In SmR1, 6 of the 14 gene mutations that increased root colonization are predicted to play a role in flagellar biosynthesis. FliF, FliG, FlhB, and FliQ are structural components of the flagellar basal body (see Fig. S2 in the supplemental material). MotA is complexed with MotB and functions as a proton channel for torque generation (29). Our testing showed that the absence of flagellar genes in the SmR1 strain conveyed an advantage for root colonization in *S. viridis* 10 days after inoculation. We also identified a poly-3-hydroxybutyrate (PHB)-related gene, a poly-3-hydroxyalkanoate depolymerase PhaZ1 (HSERO_RS08080), that enhanced colonization ability when mutated (Fig. 4). PhaZ1 is a PHB depolymerization enzyme required for the granule mobilization (30). Furthermore, mutations in the genes encoding transcriptional regulators and ABC transporters also resulted in increased root colonization by strain SmR1 (Table 1).

Insertions that impaired root association. Mutant strains corresponding to 81 DQS4^T and 114 SmR1 genes were identified as significantly reducing root colonization (i.e., fitness score ≤ -1) (Table 2; see also Table S2). Therefore, these gene functions are normally required for efficient colonization by wild-type bacteria.

Within this group of mutations, we identified two genes present in both DQS4^T and SmR1: *cheY* (DQS_RS02075 and HSERO_RS09745), which is related to chemotaxis, and *ampD* (DQS_RS17190 and HSERO_RS01880), which is involved in peptidoglycan degradation. Chemotaxis is directly involved in modulating the movement of the flagellum in response to attractants, helping bacterial orientation and active motion for survival toward favorable conditions (31–33). In addition, four other genes encoding transmembrane chemoreceptors, also known as methyl-accepting chemotaxis proteins (MCPs), were identified in *H. seropedicae*. The protein CheA (HSERO_RS15535) undergoes autophosphorylation induced by MCPs, and CheR (HSERO_RS10115) and CheW (HSERO_RS14950) acts to modulate the phosphorylation state of CheY (34) (see Fig. S3). Mutations in these genes impaired the capacity of *H. seropedicae* to colonize roots, consistent with a previous report (35).

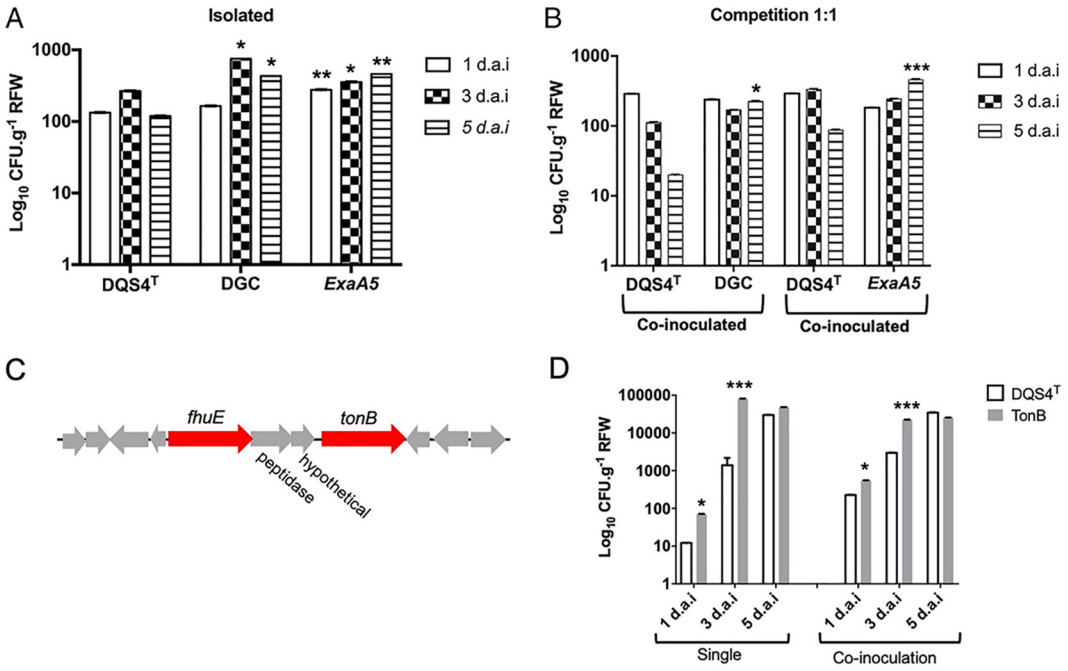

FIG 3 Root associated bacterial cells recovered from *Setaria viridis* roots at 1, 3, and 5 d.a.i. (A) Root colonization after single inoculation with wild-type DQS4^T or with the mutant lines diguanylate cyclase-DGC (DQS_RS13665) or *ExaA5* (DQS_RS19730). The data are expressed as the $\log_{10}$ CFU per g of root fresh weight (RFW). The graphs show an increase of root colonization by the mutant strains. (B) Coinoculation of *S. viridis* roots with a mixture of equal amounts of wild-type and mutant strains. (C) Map of Iron uptake cluster identified in DQS4^T by TnSeq as enhancing bacterial root attachment. (D) Root colonization of TonB mutant (DQS_RS15445) in CFU recovered from *S. viridis* roots after individual inoculation or after coinoculation with the wild type. Bars show mean averages ± the standard errors (SE) ($n = 20$). Statistical significance, determined using a Student *t* test, is indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Analysis of the DQS4^T TnSeq data identified a gene (DQS_RS09125) predicted to encode a two-component sensor histidine kinase containing a HAMP domain (histidine kinase, adenylyl cyclases, methyl-binding proteins, and phosphatases) involved in signal conversion between the transmembrane-sensing and kinase activity control (36). One assumes that this histidine kinase is reacting to an unknown factor in the rhizosphere that normally enhances root association in wild-type cells. We also observed genes involved in cell wall formation, such as *murI* (HSERO_RS07755), a glutamate racemase responsible for the conversion of L-glutamate in D-glutamate, and *murA* (HSERO_RS20355), a UDP-*N*-acetylglucosamine 1-carboxyvinyltransferase responsible for converting UDP-*N*-acetylglucosamine to

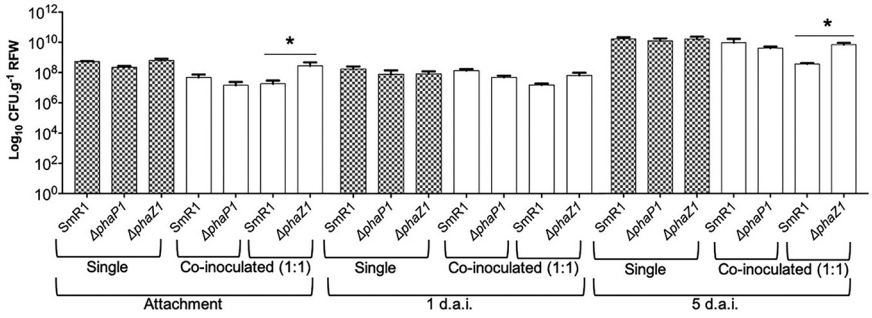

FIG 4 *Setaria viridis* root colonization by mutants defective in PHB-related genes. Mutant strains (Δ*phaP1* involved in production or Δ*phaZ1* mobilization of PHB) were recovered from root samples. The assays were performed as colonizations 30 min, 1 day, and 5 days after inoculation. The strains were inoculated individually or coinoculated in the proportion of 1:1 with wild-type SmR1. The data are expressed as the log CFU per g of root fresh tissue. Bars indicate averages ± the SE. Statistical significance, determined using a Student *t* test, is indicated by asterisks (*, $P \leq 0.01$).

TABLE 2 Partial list of mutations that impaired DQS4^T or SmR1 root fitness colonization of *S. viridis*[a]

Strain	Locus ID	Fitness score	Description
DQS4^T	DQS_RS00855	−2.70	Hypothetical protein
	DQS_RS02275	−2.33	Diguanylate cyclase
	DQS_RS01300	−2.69	RND efflux transporter, permease protein
	DQS_RS18440	−2.89	Methyltransferase domain-containing protein
	DQS_RS20485	−1.97	Putative two-component system sensor protein
	DQS_RS07570	−1.85	Putative cobalt-zinc-cadmium resistance protein
SmR1	HSERO_RS01265	−2.93	Acetyl-CoA acetyltransferase
	HSERO_RS13965	−2.79	Single-stranded DNA exonuclease
	HSERO_RS17980	−2.78	GTP-binding protein
	HSERO_RS14955	−1.99	Chemotaxis protein
	HSERO_RS00080	−1.95	Histidine kinase
	HSERO_RS20750	−1.94	Cytochrome *c* oxidase subunit I

[a]A complete list of mutations is available in Table S2 in the supplemental material. DQS4^T, *Azoarcus olearius;* SmR1, *Herbaspirillum seropedicae*.

UDP-*N*-acetylglucosamine enolpyruvate. A previous study of *H. seropedicae* reported the importance of the *mur* genes for maize root association and also showed that their expression was repressed in the presence of naringenin (37). In addition, a *N*-acetyl-anhydromuranmyl-L-alanine amidase AmpD, likely involved in the degradation of peptidoglycan by hydrolysis of muropeptides that can serve as signals for induction of β-lactamase (38–40), was identified as important for root colonization in both strains. We also identified a putative transcriptional regulator (DQS_RS12710) of catechol 2,3-dioxygenase, a flavonoid-related compound that might be involved in the degradation of aromatic compounds important for plant-bacterial interactions (41, 42). Among such compounds are flavonoids that can serve as chemoattractants for rhizobia in legumes (43, 44). Flavonoids can also mediate PGPB colonization, as reported for *H. seropedicae* colonization of *Arabidopsis* and the colonization of rice by *Serratia* spp. (45, 46). Interestingly, mutations in genes involved in PHB metabolism—including PhaP1 (HSERO_RS08150), a phasin family protein that affects PHB production, and PhbA1 (HSERO_RS01265), an acetyl-CoA acetyltransferase—decreased root colonization. The importance of PHB metabolism in bacteria during plant root colonization was reported previously in studies of *Setaria-Herbaspirillum* and legume-rhizobium symbiosis (12, 47).

Validation of candidate genes by insertional mutagenesis. To more definitively test the importance of individual genes identified in our screen, we selected candidate genes and created insertion mutations in 15 separate genes. These genes were selected to cover a diversity of putative functions representing operons containing multiple genes, as well as single genes, including those associated with both positive and negative fitness scores (Table 3). Using a gnotobiotic system, a competitive colonization assay was performed in which colonization of *S. viridis* roots by the individual mutants was measured, as well as their ability to compete for colonization when coinoculated (1:1) with the corresponding wild-type strain. Since root colonization is a dynamic process and might change over time, we evaluated competition at three different time points.

For *A. olearius* DQS4^T, we selected six genes where mutations reduced colonization, specifically a transcriptional regulator, *ybeZ*, ketoacyl ACP synthase (KAS), *peaF*, *pcm*, and *purU* (see the fitness score in Table 2).

Mutants defective in genes that resulted in reduced colonization in competition with the wild-type strain showed delayed colonization of *Setaria* roots. Mutation of the transcriptional regulator gene represents the most extreme case where colonization was completely blocked when inoculated singly onto *Setaria* roots (Fig. 5A and B; see also Fig. S4a and c). This gene encodes an AphS protein known to be involved in the phenol degradation pathway, where phenol is converted into catechol via catechol 2,3-dioxygenase (48). Analysis of *A. olearius* DQS4^T and BH72 genomes showed two

TABLE 3 Candidate genes selected for validation by gene knockout[a]

Locus ID	Gene	Fitness	Description
DQS_RS04175	*ybeZ*	−3.26	Phosphate starvation-inducible protein
DQS_RS19965	dqs_4056	–4.48	Beta-ketoacyl synthase, N-terminal domain
DQS_RS06650	*peaF*	–4.67	FAD-dependent oxidoreductase
DQS_RS04520	*pcm*	–1.38	Protein-L-isoaspartate (D-aspartate) *O*-methyltransferase
DQS_RS19155	*purU*	–2.49	Formyltetrahydrofolate deformylase
DQS_RS12710	*aphS*	−3.72	Transcriptional regulator
HSERO_RS13265	Hsero_2648	−1.17	G3E family GTPase
HSERO_RS01870	Hsero_0377	−1.75	ABC transporter permease
HSERO_RS14960	*cheY*	−2.11	Fis family transcriptional regulator
HSERO_RS08535	*opuBC*	–1.71	Glycine/betaine ABC transporter substrate-binding protein
HSERO_RS17980	*typA*	–2.78	GTP-binding protein
HSERO_RS01880	*ampD*	–2.23	*N*-Acetyl-anhydromuranmyl-L-alanine amidase
HSERO_RS07970	*purD*	−3.06	Formyltetrahydrofolate deformylase
HSERO_RS03440	*exbD*	−2.43	Biopolymer transporter ExbD
HSERO_RS03445	*exbB*	−2.61	Biopolymer transport transmembrane protein

[a]The descriptions are based on eggNog annotation and UniProt. DQS4^T, *Azoarcus olearious*; SmR1, *Herbaspirillum seropedicae*.

operons potentially involved in the degradation of aromatic compounds located adjacent to the *aphS* gene (Fig. 6). However, consistent with the TnSeq results, when coinoculated with the wild-type DQS4^T, the mutant reduced colonization more modestly (see Fig. S4b and d). This result suggests the need for an unknown metabolite or signal

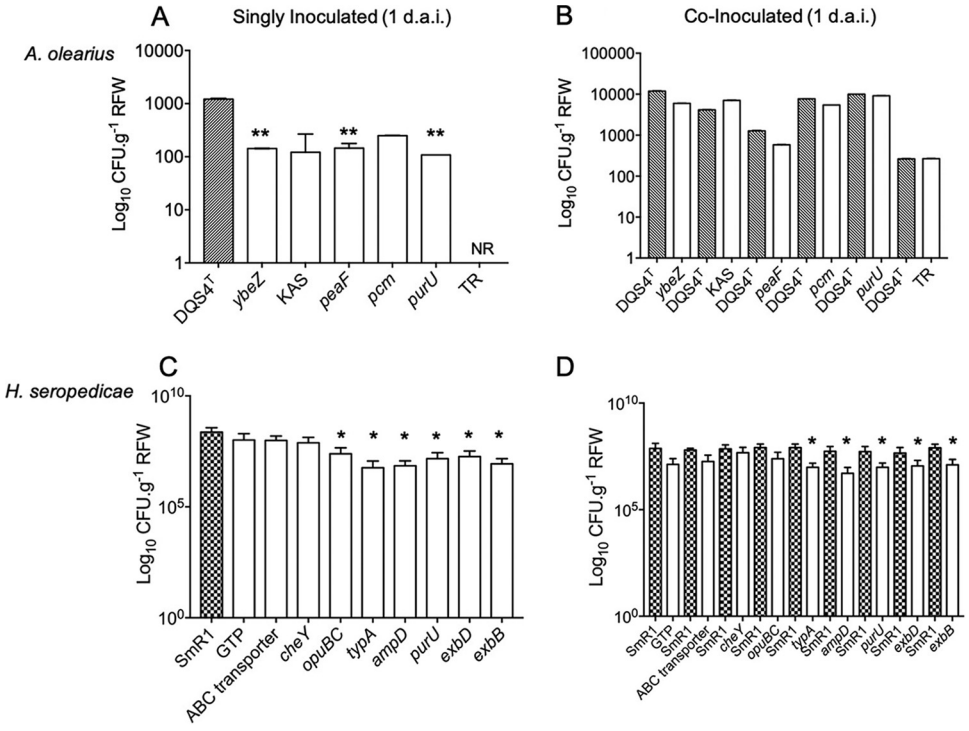

FIG 5 Candidate genes selected for validation during *S. viridis* root colonization. Root colonization by *Azoarcus olearius* DQS4^T or *Herbaspirillum seropedicae* SmR1 wild type and mutants defective in genes that decreased bacterial root colonization. (A) Root colonization by DQS4^T wild type (solid gray bars) and six mutant strains (white bars) when inoculated individually, recovered at 1 d.a.i. (B) Coinoculation of *S. viridis* roots with a mixture of equal amounts of wild type DQS4^T and mutant strains. (C) Root colonization of SmR1 wild type and nine mutant strains at 1 d.a.i. when inoculated individually. (D) Coinoculation with a mixture of equal amounts of wild-type SmR1 and mutant strains. The data are expressed in log of CFU per g of root fresh tissue. Bars indicate mean averages ± the SE ($n = 20$). Statistical significance, determined using a Student *t* test, is indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). NR, not recovered; KAS, ketoacyl ACP synthase; TR, transcriptional regulator; ABC, ABC transporters.

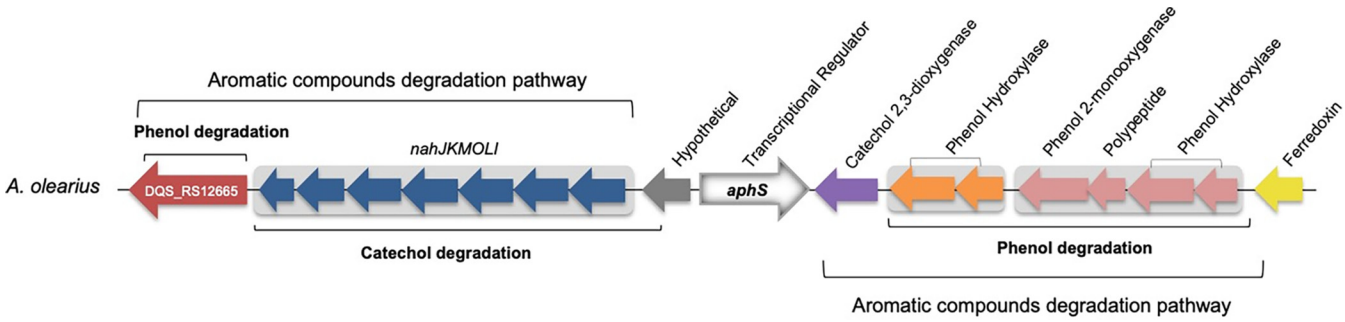

FIG 6 Operon organization of aromatic metabolism in *Azoarcus olearius* DQS4^T. The transcriptional regulator *aphS* (DQS_RS12710) identified by TnSeq regulates genes related to phenol degradation and utilization. Genes and operons are indicated by arrows; use of the same color within a gray shade indicates genes that belong to an operon. DQS_RS12665 indicates the degradation protein meta in *A. olearius*.

that is produced by the wild type that can compensate for its loss in the mutant. As for the other mutants that impaired *A. olearius* DQS4^T colonization *ybeZ*, ketoacyl ACP synthase (KAS), *peaF*, *pcm*, and *purU*, all showed a significant reduction in colonization that was most pronounced 3 and 5 days after inoculation both when inoculated individually or in competition with the wild-type strain (see Fig. S4a and b).

Interestingly, *H. seropedicae* SmR1 genes related to PHB metabolism appeared to affect fitness by enhancing or decreasing root colonization as shown in Fig. 4. Among the nine selected genes where mutations impaired colonization by *H. seropedicae* (Table 2), six mutants—specifically the *opuBC*, *typA*, *ampD*, *purD*, *exbD*, and *exbB* mutants—showed a significant reduction in root colonization either when inoculated separately or coinoculated with the wild type (Fig. 5C and D; see also Fig. S5a and d).

DISCUSSION

Applying the transposon mutagenesis sequencing (TnSeq) approach to both *A. olearius* DQS4^T and *H. seropedicae* SmR1 revealed many genes required for these bacteria to competitively colonize *Setaria viridis* roots.

Our experiments identified 89 and 130 genes where mutations significantly affected the ability of *A. olearius* or *H. seropedicae*, respectively, to colonize *S. viridis* roots, including some genes previously reported to play a role in the plant-microbe associations. This result alone argues that root colonization is not a simple process but one that involves a variety of bacterial functions. General gene classes include those involved in cell wall biosynthesis, motility, chemotaxis and defense, and amino acid metabolism (19, 49–51).

Colonization assays with the 15 candidate genes selected for further confirmation by insertional mutagenesis showed a strong correlation with the results obtained by TnSeq, giving confidence that most if not all of the genes identified are likely important for root colonization by these PGPB strains.

Genes where mutations benefited root colonization can be assumed to normally play a role in suppressing colonization in wild-type cells. Among such genes in *A. olearius* DQS4^T is a homolog of the BH72 *exaA5* gene (azo3865), predicted to encode a pyrroloquinoline quinone-dependent alcohol dehydrogenase (DQS_RS19730) involved in methanol oxidation. Consistent with these findings, previous reports showed that mutation of ADH genes inhibited competitive colonization of rice roots by *A. olearius* BH72 (21, 52). Similarly, *Methylobacterium* spp. mutants defective in methanol oxidation were less competitive for colonization of *Medicago truncatula* roots when coinoculated with wild-type cells (53, 54). These data suggest that methanol metabolism is important for bacterial growth on the root surface and, perhaps more importantly, colonization is a very dynamic and likely heavily influenced by the overall microbial community.

We observed genes that increased fitness scores clustered within an operon presumably involved in iron uptake in *Azoarcus*. These genes are predicted to encode an outer membrane, ferric coprogen protein FhuE (DQS_RS15430), and a TonB-dependent siderophore receptor (DQS_RS15445), both of which were previously implicated in the

ability of this bacterium to colonize roots (9, 14). Bacterial iron uptake is complex, perhaps involving multiple bacterial processes, and can also be coopted by plant-encoded mechanisms. Mutation of these genes increased fitness values conveying a phenotypic advantage during root colonization, although less competitive than the wild type. Given the general role that iron availability plays in the ability of microorganisms to thrive and compete in virtually any environment, it is not surprising that iron uptake is also a crucial function for root colonization (17, 55). Recently, analysis of iron content in maize treated with the PGPB *A. brasilense* revealed a significant increase in total iron accumulation in seeds and higher yield (56), suggesting that PGPB can contribute to the iron metabolism of the host plant.

Previous studies demonstrated an important role for bacterial genes involved in motility for both endophytic and rhizosphere colonization of host roots (38, 57). Many genes involved in cell motility were among the common COG categories that appear to provide a fitness advantage for root colonization by SmR1, specifically mutations in genes related to flagellum assembly. We showed previously that an *H. seropedicae* mutant in the flagellar regulatory gene *fliA* was unable to endophytically colonize *S. viridis* roots, although this mutation did not affect rhizosphere colonization (12). *fliA* encodes the sigma factor σ28 RNA polymerase that mediates the transcription of genes involved in motility and flagellar synthesis (58). It is hard to imagine how the loss of motility *per se* could enhance root colonization. However, bacterial flagella can be recognized by specific receptors in plant cell membranes and activate a cascade of immune responses controlling bacterial infection (59–61). Transcriptome analysis of SmR1 attached to wheat roots showed that the flagellar gene cluster was downregulated, suggesting that the bacteria might switch to a twitching type of motility mediated by type IV pili (62).

Under certain environmental stresses or nutritional conditions, bacteria can use different sources of energy for survival, including mobilization of polymers such as polyhydroxyalkanoates (PHA). PHB is the PHA produced by bacteria. The PHB granules act as carbon storage that can be mobilized under different conditions. We found that disruption of PHA depolymerase, encoded by the *phaZ1* gene, enhanced bacterial colonization when inoculated individually or in competition with the wild type. According to Silveira Alves et al. (12), plant biomass was significantly reduced in *S. viridis* colonized by Δ*phaZ1* mutant despite colonizing roots to the same level as the wild-type strain. In contrast, we identified PhaP1 encoding a phasin involved in the PHB production (62–66), where deletion of Δ*phaP1* affected fitness negatively, reducing root colonization. Corroborating our findings, an increase in gene expression of the phasin genes was reported during wheat root colonization (62).

Many genes involved in peptidoglycan and lipopolysaccharide (LPS) biosynthesis were predicted to impair bacterial colonization of plant roots. For instance, mutation of LPS biosynthetic genes or the addition of exogenous *N*-acetylglucosamine was previously shown to impair *H. seropedicae*-maize root association (67). In studies of rice roots colonized by *Azoarcus* BH72, mutation of an endoglucanase (enzyme that cleaves cellulose) reduced root colonization, suggesting its importance for successful host cell invasion (68, 69). We demonstrated that a mutation in a transcriptional regulator (*aphS*) involved in aromatic compound degradation completely impaired root colonization during single inoculation. AphS was predicted to regulate genes important for phenol degradation in *A. olearius* BH72 (48).

Only two genes, *cheY* involved in chemotaxis and *ampD* involved in peptidoglycan cell wall recycling (39, 70), affected colonization of both strains. Our data suggest that bacterial chemotaxis provides a competitive advantage to wild-type cells during colonization of the plant root tissue. This system is well characterized in several motile bacterial species, such as *E. coli* and beneficial bacteria such as *Azospirillum brasilense*, *S. meliloti*, and others (31, 71–75).

In summary, our data indicate that, rather than a single or small subset of crucial functions, each strain uses differing functions for colonization, reflecting the unique

characteristics of each bacterium. Given that our study was focused on bacterial root colonization, mutations related to soil survival were not considered. However, for *Azoarcus* a deeper investigation of mutations that affected survival in soil could be useful in explaining the different lifestyle and adaptions of each bacterium to their environment, especially considering that *Azoarcus* sp. BH72, closely related to *A. olearius* DQS4^T, is a strict endophyte and has not been reported to survive without a host (76).

In summary, similar to most plant-microbe interactions, PGPB-plant interactions are complex and reflect the ability of the plant host and bacterial symbiont to profoundly influence the metabolism of the other. The fact that PGPB have broad host ranges and can enhance crop yield under field conditions has contributed to a continuing interest in using PGPB inoculants in agriculture. This study provides insights to better understand those gene functions involved in PGPB-host interaction and hopefully will contribute to the further development of PGPB inoculants for an efficient, sustainable agriculture.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Azoarcus olearius* DQS4^T (Sm^r Nal^r) and *Herbaspirillum seropedicae* SmR1 (Sm^r) were used as bacterial model systems for this study. Both strains were grown in NFb-HPN (high phosphate and nitrogen) medium containing malate as carbon source (77, 78) supplemented with NH_4Cl (10 mmol liter^{-1} [DQS4^T] and 20 mmol liter^{-1} [SmR1]) according to the strain's requirements. The bacterial cultures were grown at 37°C for DQS4^T and 30°C for SmR1, with shaking at 130 rpm overnight.

***Setaria viridis* seed sterilization and growth conditions.** *Setaria viridis* A10.1 seeds were treated with 5 ml of sulfuric acid for 15 min to break dormancy and washed with running water for 1 min. Upon washing, the seeds were surface sterilized with 1% bleach plus 0.1% Tween 20 (vol/vol) for 3 min and then rinsed three times with sterile distilled water. Sterile seeds were sown onto plates containing one half Murashige-Skoog medium with 5% sucrose (wt/vol) and 1% Phytagel (wt/vol). Plates were placed in the dark for 24 h and then 2 days in the light at 30°C for germination. After germination, 20 seedlings of similar sizes were transferred to a pot containing sterilized Turface (Turface MVP) and vermiculite in a 3:1 proportion and grown for 5 days prior to inoculation. Four biological replicates were performed, including soil controls without plants. Plants were watered every day, and an additional 0.2% malate solution was added to the soil control as a carbon source to enhance bacterial survival.

Transposon mutant library construction. We applied two different approaches to generate mutant libraries for each strain, using engineered Tn*5* transposon delivery vectors. (i) *A. olearius* DQS4^T was conjugated with *E. coli* harboring pJG714, which is described in (79). Around 130,000 transposants were generated by selection on kanamycin (50 μg ml^{-1}). The mutant library was harvested from plates and stored in 10% glycerol at −80°C for further TnSeq experiments. (ii) An *H. seropedicae* SmR1 library was constructed as previously described (18, 80) using a random barcode (RB-TnSeq) method (16). Cell aliquots of 1 ml (optical density at 600 nm [OD_{600}] of 1) were stored in 10% glycerol at −80°C until the TnSeq experiments were performed.

Mutant library competitive fitness assay on plant roots. A single aliquot of the DQS4^T mutant library was thawed and 50 μl was inoculated into 50 ml of modified Luria-Bertani (LB) medium containing 5 g liter^{-1} tryptone, 5 g liter^{-1} yeast extract, 3 g liter^{-1} NaCl, and 0.5 g liter^{-1} $MgSO_4s^27H_2O$ supplemented with kanamycin. For SmR1, glycerol stocks of 1-ml aliquots of the mutant library were inoculated into 50 ml of LB medium with kanamycin. The cultures were grown until reaching an OD_{600} of 1.0, pelleted, and washed prior to dilution to 2×10^9 cells/ml (DQS4^T) or 2×10^8 cells/ml (SmR1) in modified Hoagland's solution without nitrogen source (81). Then, 50 ml of diluted DQS4^T or 10 ml of SmR1 culture was added directly onto the soil in each pot containing 20 plants or soil control pots. A plate was placed on the bottom of the pots in order to collect the flowthrough culture, and the plants were reinoculated after 15 min. The plants were kept in a growth chamber (Conviron) at 25°C with 16-h light/8-h dark cycle for 15 days. After 15 days, mutants were recovered from *S. viridis* roots by removing the 20 plants from the soil. Using a sterile surface, a 3:1 vermiculite potting mix allowed for the removal of the plants from the potting mix with few adhering particles. Roots were immersed into 100 ml of BRM medium incubated at 37°C for DQS4^T or NFb-HPN-malate incubated at 30°C for SmR1, both containing kanamycin (50 μg/ml), and then incubated for 6 h under 120 rpm agitation. After 6 h of agitation, 50-ml portions of the culture were filtered through sterile filter paper to eliminate any soil debris and transferred to a new flask with fresh medium (Km^r) incubated at 37°C or 30°C, according to each strain, followed by shaking at 120 rpm overnight. The cultures were then centrifuged for 2 min at 14,000 rpm. The supernatant was discarded; the pellets were immediately frozen in liquid nitrogen and then stored at −80°C prior to sequencing library preparation. Soil culture was filtered after 3 h of incubation to settle down soil particles to enable their separation from the culture.

TnSeq library preparation. For *Azoarcus olearius* DQS4^T, a bacterial culture at mid-log phase grown at 37°C was collected for DNA extraction. Cells were pelleted by centrifugation and immediately frozen at −80°C until DNA extraction. Genomic DNA was extracted, and transposon insertion junctions were selectively amplified, as described previously (79). Insertion junction libraries were multiplexed and sequenced using the Illumina HiSeq 2500 system. For *Herbaspirillum seropedicae* SmR1, libraries were sequenced on either the HiSeq 2000 or HiSeq 2500 system (Illumina) to map a greater fraction of the

mutant population. Genomic DNA was extracted, and barcodes were amplified as described previously (16, 18, 80).

Analysis of transposon insertion impact on bacterial fitness. For *A. olearius*, TnSeq reads were mapped to the reference genome, and genes received fitness values representing the relative abundance of insertions across conditions. For *H. seropedicae* SmR1, we calculated gene fitness scores based on the barcode abundance of the individual strains in the library. The fitness values represent the count in each sample relative to the time zero sample (for details, see reference 6). Differences in fitness values with a *P* value of $\leq$0.05 were considered statistically significant and categorized into two groups based on their fitness score: (i) enhanced fitness ≥ 1 or (ii) decreased fitness ≤ -1.

Confirmation of gene functionality by site-directed mutagenesis. The suicide plasmid pJG194 carrying Km^r (82) was used to generate targeted bacterial gene insertion mutations in various candidate genes via single crossover. Amplicons (~500 bp) for internal gene fragments were cloned into plasmid pJG194 using HindIII and EcoRI restriction sites (see Table S1 in the supplemental material). Bacterial template gDNA was extracted by using a DNeasy Blood & Tissue (Qiagen) according to the protocol for Gram-negative bacteria. Amplification reactions were carried out using Phusion high-fidelity DNA polymerase (Thermo Fisher) under the following cycling conditions: 98°C for 1 min; 30 cycles 98°C for 20 s, 57°C for 30 s, and 72°C for 30 s; and a final extension of 72°C for 10 min, 4°C. PCR products were verified on a 1% electrophoresis agarose gel for the correct 500-bp size. The correct products were purified using Wizard SV gel and PCR cleanup system (Promega) and then digested with FastDigest HindIII and EcoRI for 20 min each. Samples were ligated with T4 ligase at 4°C overnight. Ligation products were transformed into *E. coli* EC100 competent cells (Epicentre, Madison, WI) by heat shock (10 min on ice, 45 s at 42°C, and 1 min on ice) and then added to 200 μl of LB medium, followed by shaking at 37°C for 1 h prior plating onto LB+Km^r. The inserts of the generated plasmids were confirmed by PCR and DNA sequencing prior to transformation into *E. coli* ST18 (83) or S17.1 (84) for mating with the wild-type DQS4^T or SmR1 strain, respectively. Mutants were confirmed to have the desired gene disruptions by PCR analysis and DNA sequencing.

Competition assay *in planta*. To assess bacterial gene function in plant colonization, we performed a colonization assay using *Setaria viridis* seedlings grown hydroponically. Seeds were germinated on plates as described above and transferred to a glass tube containing 20 ml of sterile modified Hoagland's solution and 8 g of polypropylene beads. Seedlings were inoculated with 1 ml of bacterial culture (10^{-6} cells ml^{-1}) of the wild-type strain or mutant strain separately or in a 1:1 proportion. Quantification of colonization was performed by recovering the attached bacteria from the roots ($n = 20$) at 1, 3, and 5 d.a.i. Briefly, seedlings were carefully removed from the glass tubes and placed in a 2-ml tube. The roots were weighed, and then 1 ml of 0.9% NaCl was added to each tube. To recover the surface-attached bacteria, tubes were vortexed at high speed for 1 min, and the solution containing suspended bacteria was serially diluted. The dilutions were plated onto NFb-HPN malate medium containing appropriate antibiotics, including kanamycin for the targeted gene disruption mutants, and then incubated at 30°C for 24 h prior to colony counting and conversion to CFU g^{-1} of fresh tissue.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, TIF file, 2.1 MB.

FIG S2, TIF file, 2.6 MB.

FIG S3, TIF file, 2.4 MB.

FIG S4, TIF file, 2.3 MB.

FIG S5, TIF file, 1.8 MB.

TABLE S1, DOCX file, 0.02 MB.

TABLE S2, XLSX file, 0.02 MB.

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We declare that we have no conflicts of interest.

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