AMERICAN SOCIETY FOR MICROBIOLOGY | Journal of Bacteriology®

RESEARCH ARTICLE

Transcription Factor PecS Mediates Agrobacterium fabrum Fitness and Survival

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ABSTRACT The transcriptional regulator PecS is encoded by select bacterial pathogens. For instance, in the plant pathogen Dickeya dadantii, PecS controls a range of virulence genes, including pectinase genes and the divergently oriented gene pecM, which encodes an efflux pump through which the antioxidant indigoidine is exported. In the plant pathogen Agrobacterium fabrum (formerly named Agrobacterium tumefaciens), the pecS-pecM locus is conserved. Using a strain of A. fabrum in which pecS has been disrupted, we show here that PecS controls a range of phenotypes that are associated with bacterial fitness. PecS represses flagellar motility and chemotaxis, which are processes that are important for A. fabrum to reach plant wound sites. Biofilm formation and microaerobic survival are reduced in the pecS disruption strain, whereas the production of acyl homoserine lactone (AHL) and resistance to reactive oxygen species (ROS) are increased when pecS is disrupted. AHL production and resistance to ROS are expected to be particularly relevant in the host environment. We also show that PecS does not participate in the induction of vir genes. The inducing ligands for PecS, urate, and xanthine, may be found in the rhizosphere, and they accumulate within the plant host upon infection. Therefore, our data suggest that PecS mediates A. fabrum fitness during its transition from the rhizosphere to the host plant.

IMPORTANCE PecS is a transcription factor that is conserved in several pathogenic bacteria, where it regulates virulence genes. The plant pathogen Agrobacterium fabrum is important not only for its induction of crown galls in susceptible plants but also for its role as a tool in the genetic manipulation of host plants. We show here that A. fabrum PecS controls a range of phenotypes, which would confer the bacteria an advantage while transitioning from the rhizosphere to the host plant. This includes the production of signaling molecules, which are critical for the propagation of the tumor-inducing plasmid. A more complete understanding of the infection process may inform approaches by which to treat infections as well as to facilitate the transformation of recalcitrant plant species.

KEYWORDS PecS, Arabidopsis, virulence gene, MarR, urate, fitness

The multiple antibiotic resistance regulators (MarR) constitute a large family of transcriptional regulators that are ubiquitous in both eubacteria and archaea, and they are thought to have originated before the divergence of these kingdoms (1). The original marR gene, first characterized in Escherichia coli, is part of an operon that encodes proteins that confer resistance to antibiotics, phenolic compounds, organic solvents, and household disinfectants (2). As disease-causing bacteria transition from free-living to pathogenic phases, the need arises for a proper coordination of the changing gene expression, including the integration of several regulatory circuits that control cell-cell communication or sense environmental conditions (3). MarR proteins play a critical role in bacterial survival and virulence, including during plant-pathogen interactions, by responding to molecular cues. Typically, the binding of cognate ligands to the MarR

Editor Anke Becker, Philipps-Universitat Marburg Fachbereich Biologie

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The authors declare no conflict of interest.

Received 23 December 2022
Accepted 19 May 2023
Published 14 June 2023

protein leads to protein conformational changes that, in turn, prevent DNA binding, thereby leading to the differential expression of target genes (1).

PecS is a member of the MarR protein family, and it was originally characterized as a master regulator of virulence gene expression in the Enterobacterium Dickeya dadantii, which is a soft, rot-causing phytopathogen that infects a range of plant species (4, 5). Global transcriptome profiles in D. dadantii wild-type and pecS mutant strains during the early colonization of leaf surfaces and in leaf tissue collected just before the onset of symptoms show that the PecS regulon consists of over 600 genes (5). For example, D. dadantii PecS controls the expression of pectinase genes that are essential for infectivity and disease progression, and it represses the biosynthesis of a blue pigment that is known as indigoidine and functions as an antioxidant (4, 6). This global regulator also regulates a few dozen genes that encode regulatory proteins, demonstrating that PecS is at or near the top of a major regulatory cascade that governs adaptation to growth in planta (5).

The D. dadantii pecS gene is encoded divergently to pecM, which encodes an efflux pump for indigoidine (6–8), and the expression of both genes is under the control of PecS. This pecS-pecM gene pair is conserved in several other plant pathogens, such as Agrobacterium fabrum and Pectobacterium atrosepticum (9, 10), and it has also been identified in the human pathogens Klebsiella pneumoniae (11) and Vibrio vulnificus (12). For instance, in K. pneumoniae, PecS regulates the expression of type 1 fimbriae indirectly and negatively, possibly through the nucleoid-associated protein H-NS; type 1 fimbriae are crucial virulence factors in mediating Klebsiella urinary tract infections (11).

The Gram-negative, soilborne A. fabrum is a plant pathogen that is the causative agent of crown galls, a neoplastic plant disease. It is an intensively studied plant pathogen because of its importance as a tool in genetic engineering, hence its agricultural implications. The infection process is governed by both chromosomally and plasmid-encoded virulence factors as well as by the tumor inducing, plasmid-borne oncogenes and the transfer-DNA (T-DNA) (13). A. fabrum induces the formation of tumors by inserting the T-DNA into the nuclear DNA of host plants, thereby modulating plant cell division (13). This transfer of DNA is the only known natural example of interkingdom DNA transfer.

The expression of chromosomal genes is key to several cellular processes, such as flagellar motility, chemotactic responses, biofilm formation, plant attachment, and vir gene induction, all of which are critical early steps in crown gall formation and bacterial survival. For instance, the expression of the che cluster (chemotaxis) allows A. fabrum to be attracted to plant-derived chemicals in the rhizosphere (14), whereas the product of the virulence gene chvE is required for vir gene induction by sugars (15).

Bacteria attempting to infect a would-be plant host are also met with a range of host defenses. One such defense is the production of reactive oxygen species (ROS) (16). The enzyme xanthine dehydrogenase participates in purine metabolism by converting hypoxanthine to xanthine and xanthine to urate. It also participates in ROS production when O_2 is the final acceptor for electrons. Consequently, both urate and xanthine accumulate in plant cells during infection (17). Both urate and xanthine have been shown to bind A. fabrum PecS and to cause attenuated DNA binding (10). Accordingly, the accumulation of urate and xanthine in an infected plant could serve as a signal for colonization and elicit the differential expression of the PecS regulon. In addition, the roots release an abundance of organic compounds into the rhizosphere, including purines (18), suggesting that the PecS regulon may also be induced in the rhizosphere.

Using an A. fabrum pecS disruption strain, we show that A. fabrum PecS is implicated in many of the steps preceding plant transformation, including chemotactic movement, biofilm formation, and stress responses. These data suggest that A. fabrum PecS functions to promote bacterial fitness during plant infections.

RESULTS

A. fabrum PecS represses the pecM gene. A. fabrum PecS is expected to control the expression of its own gene and the divergently oriented pecM gene by direct

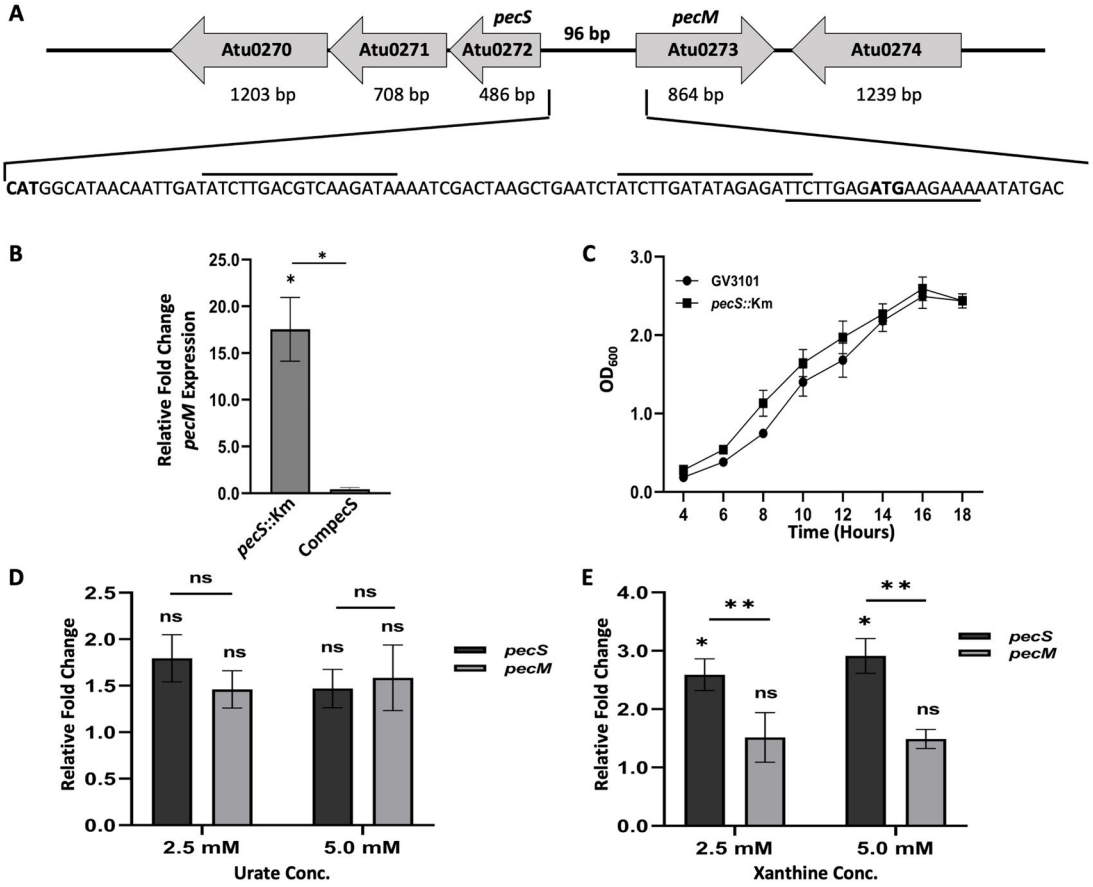

FIG 1 Expression of pecM is repressed by A. fabrum PecS. (A) Genetic organization of pecS and adjacent genes (not to scale). Open arrows represent the genes. pecS and pecM are separated by a 96 bp intergenic region. The sequence of the intergenic region is shown with the PecS binding sites overlined or underlined. Start codons are in boldface. (B) The relative abundance of pecM transcript levels in pecS::Km and CompecS strains, compared to wild-type cells. The mRNA levels were measured with RT-qPCR, and the relative abundance was calculated, relative to wild-type cells, using the comparative C_T method. Asterisks represent statistically significant differences, compared to wild-type cells, unless indicated otherwise, based on a Student's t test. *, P , 0.05. (C) Growth of GV3101 and pecS::Km strains over an 18 h period in LB. All strains were cultivated at 28°C with shaking. To determine the cell density, aliquots were collected from the cultures at the specified times. The data represent the means from three biological replicates with the standard deviations. (D) The relative fold change in pecS and pecM transcript levels in A. fabrum after exposure to 2.5 mM and 5.0 mM urate. (E) The relative fold change in pecS and pecM transcript levels in A. fabrum after exposure to 2.5 mM and 5.0 mM xanthine. The relative mRNA transcript levels of both genes and rpoA (reference control gene) were measured by RT-qPCR, relative to cultures supplemented with an equal volume of 0.4 M NaOH (the solvent for the purines). The error bars represent the standard deviations from three replicates. ns, not significant; *, P , 0.05; **, P , 0.01.

binding to a 96 bp intergenic pecS-pecM region (Fig. 1A), thereby repressing both genes (10). According to MicrobesOnline (19), pecS is predicted to be a part of an operon encoding a GntR family transcription factor (Atu0271) and a muconate lactonizing enzyme family protein (Atu0270), both of which are proteins of unknown function. It was previously determined, based on DNase I footprinting and the titration of PecS under stoichiometric conditions, that two PecS dimers protect two overlapping palindromic sequences on the forward strand (with respect to pecM), which is a sequence that includes the start codon of pecM (Fig. 1A) (10), whereas a single PecS dimer protects a palindrome in the pecS promoter (Fig. 1A). To verify the predicted regulatory mechanism, the gene encoding PecS was disrupted by plasmid insertion to generate strain pecS::Km, and the gene disruption was complemented with plasmid-borne pecS that was expressed from an arabinose-inducible promoter (a strain referred to as CompecS). The pecM transcript levels were determined using RT-qPCR (Fig. 1B). Consistent with expectations, pecM expression was increased approximately 17-fold in the pecS::Km strain, whereas complementation with plasmid-encoded pecS completely restored repression

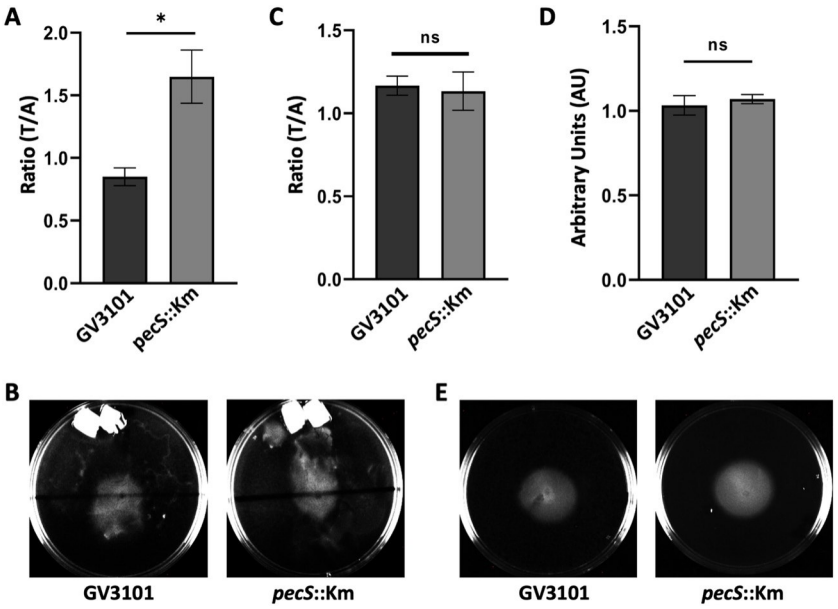

FIG 2 Chemotactic agar phenotypes of GV3101 and pecS::Km. (A) Quantified T/A ratio of wild-type and pecS::Km movement toward (T) or away (A) from onion explants. (B) Swim plate images showing the movement of wild-type and pecS::Km cells in the presence of onion explants (top of plate; LB medium). (C) Quantified T/A ratio of wild-type and pecS::Km movement toward (T) or away (A) from acetosyringone. (D) Quantified swim ring diameter of wild-type and pecS::Km cells in the presence of sucrose attractant (AT medium). (E) Swim plate images showing the movement of wild-type and pecS::Km cells in the presence of sucrose attractant. The plates were supplemented with 0.3% agar. The cells were incubated for 48 h. The values are the averages of three swim plates per strain. The asterisks represent statistically significant differences, based on a Student's t test. ns, not significant; *, P , 0.05.

(Fig. 1B). Both the wild-type and pecS::Km grew to stationary phase after culture for 16 h in Luria-Bertani (LB) medium, and they had similar doubling times of 3.5 h (Fig. 1C). This result shows that a pecS disruption does not impact the growth of A. fabrum in rich media.

Xanthine differentially induces the expression of pecS and pecM. It has been reported that urate is a ligand for PecS in A. fabrum (10). The urate-mediated upregulation of pecS and pecM has likewise been reported in both Streptomyces coelicolor (20) and K. pneumoniae, where the increased expression of the two genes was reported after the addition of 5.0 mM urate (11). Xanthine, which is a precursor of uric acid in the purine salvage pathway, also binds directly to PecS, thereby resulting in the attenuation of DNA binding (10). Therefore, we investigated the expression of pecS and pecM genes in the presence of urate and xanthine. Whereas the pecS and pecM mRNA transcript levels did not significantly increase upon the addition of 2.5 or 5 mM urate to the growth medium (Fig. 1D), xanthine significantly and selectively increased pecS expression (Fig. 1E).

Motility and chemotactic movement is enhanced in pecS::Km. Several studies have reported chemotactic movement by Agrobacterium toward phenolic plant exudates and sucrose as an important early step in host colonization (21). To determine whether PecS regulates chemotactic movement, we compared chemotactic movements on a 0.3% agar AT minimal medium in the presence of sucrose, acetosyringone, or an onion explant. The movement toward acetosyringone or the onion explant was measured by comparing the ratio of the movement toward the potential attractant over the movement away from it (T/A). In the presence of the onion explant, the pecS::Km moved more toward the source of the attractant with a higher T/A ratio of 1.6, compared to a T/A ratio of 0.9 for the wild-type (WT) (Fig. 2A and B). Acetosyringone did not induce differential motility of the two strains (Fig. 2C). Sucrose also plays a significant role in wound signaling; however, no differ-ence in movement between wild-type and pecS::Km strains was observed in the presence of sucrose, as measured by the comparable ring diameter for both (Fig. 2D and E).

Bacterial motility generally plays a significant role in host colonization (21). Therefore, we assayed for swimming motility on a 0.3% agar plate; compared to the wild-type,

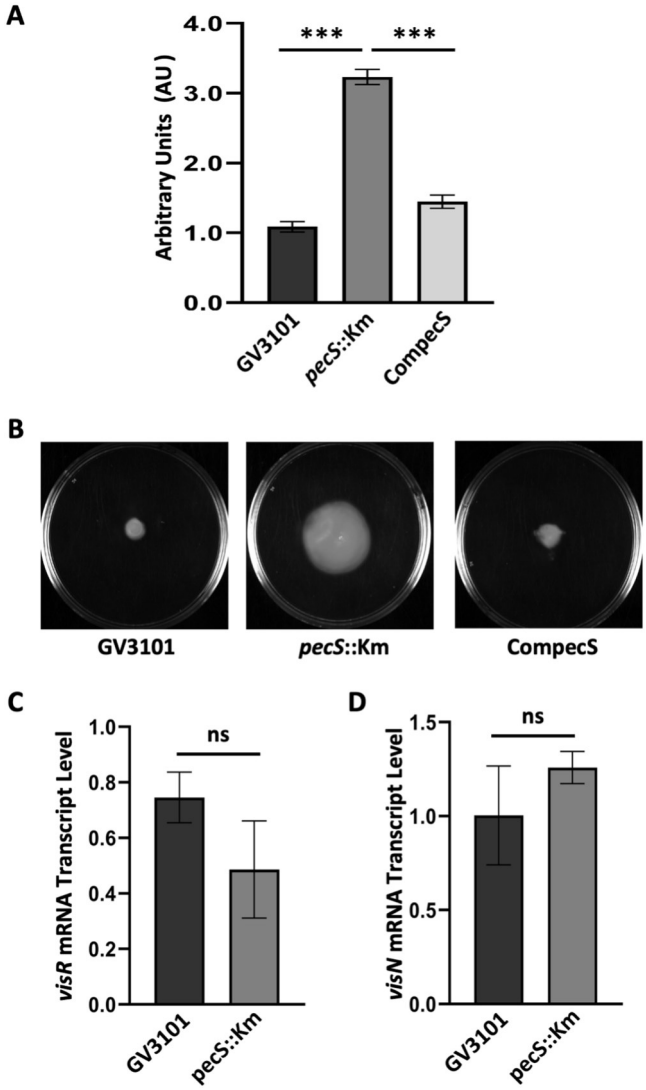

FIG 3 Swimming motility is increased in pecS::Km cells. LB plates were supplemented with 0.3% agar. (A) Quantified swim ring diameter of GV3101, pecS::Km, and CompecS cells. The values are the averages of three swim plates per strain. (B) Representative swim plates of GV3101, pecS::Km, and CompecS strains. The cells were incubated for 48 h. (C and D) Abundance of visR and visN mRNA, relative to rpoA (reference gene) in GV3101 and pecS::Km cells. The asterisks represent statistically significant differences, based on a Student's t test. ns, not significant; ***, P , 0.001.

greater motility was evident for the pecS::Km strain, with a swim ring diameter that was threefold greater than that of the wild-type cells after 48 h (Fig. 3A and B). A normal swim phenotype was observed by the strain harboring the plasmid-borne copy of pecS. These data clearly implicate PecS in controlling motility. The majority of genes associated with chemotaxis are encoded within a single gene cluster (22, 23), which is a gene clus-ter that also encodes the two master regulators VisN and VisR (Atu0524-0525). Since these transcription factors promote flagellar motility and suppress biofilm formation, we examined their expression. As shown in Fig. 3C and D, pecS::Km cells did not exhibit significantly increased expression of visR and visN.

Biofilm formation is impaired in the pecS::Km strain. A. fabrum forms biofilm on plant tissues and abiotic surfaces (21). The formation of biofilm is an important key step in crown gall disease, and biofilm maturation can be controlled at multiple points via different regulatory pathways that respond to changing environmental cues (24). We investigated the possible role of PecS in biofilm formation by using a crystal violet assay. Compared to wild-type cells, the pecS::Km strain showed a deficiency in biofilm

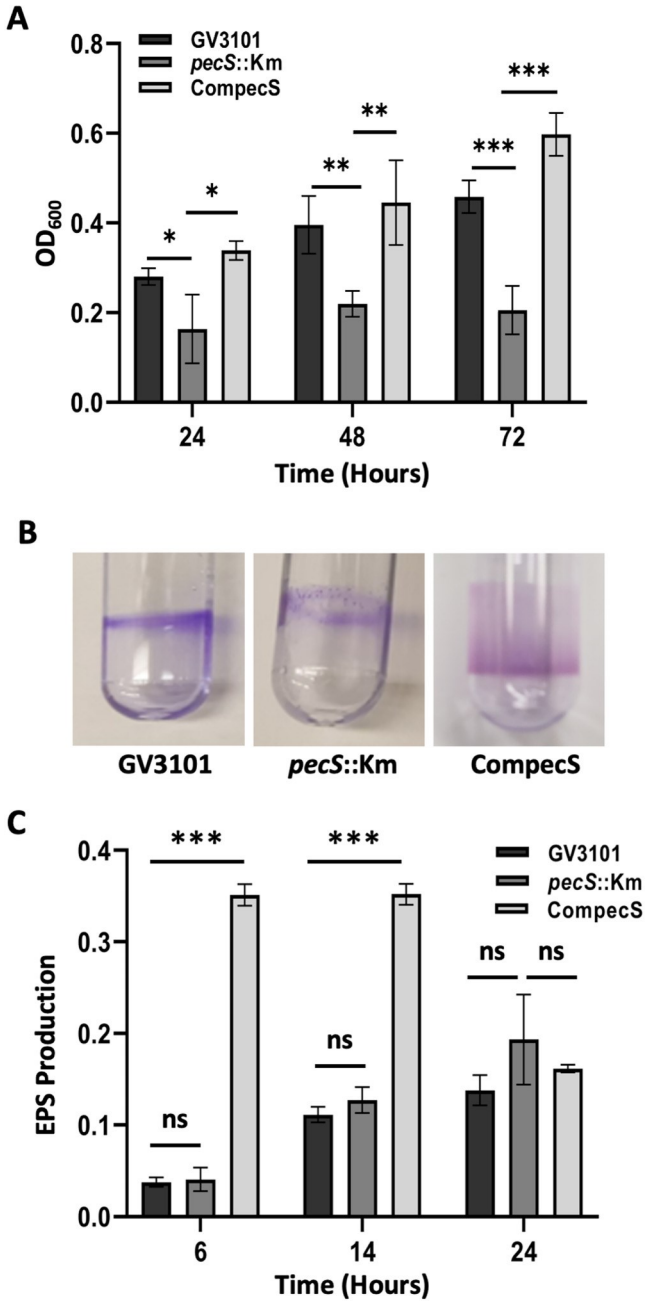

FIG 4 Biofilm formation is decreased in the pecS::Km strain. (A) Quantitation of biofilm formation in a static culture. (B) Representative crystal violet-stained biofilms. (C) Quantitation of exopolysaccharide production with 5 mL of supernatant from the indicated growth time points. The data represent the mean and SD from three biological replicates. The asterisks represent statistically significant differences, compared to wild-type cells (GV3101), based on a Student's t test. ns, not significant; *, P , 0.05; **, P , 0.01; ***, P , 0.001.

formation, and this deficiency was observed after incubation for one to three days (Fig. 4A and B). Biofilm formation was restored in the complemented strain for which the biofilm was not confined to the immediate area of the air-liquid interface; instead, it appeared to spread across the surface (Fig. 4B).

Exopolysaccharides (EPS) are extracellular macromolecules that are especially effective in protecting cells from osmotic stress, desiccation, phagocytosis, cell recognition, and hazardous substances, and they are important during biofilm maturation (25). Therefore, we investigated exopolysaccharide production, and our data showed no

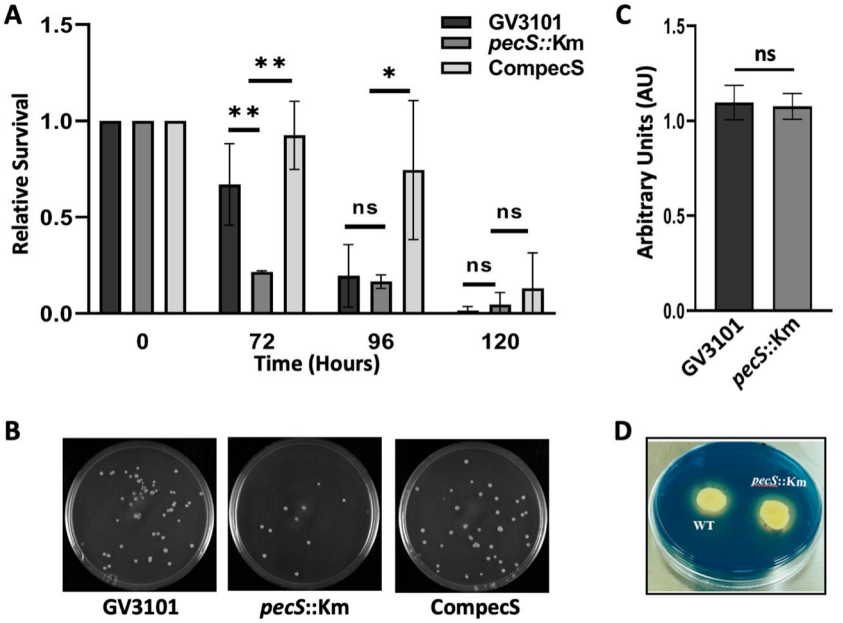

FIG 5 Phenotypes linked to microaerobic survival and siderophore production. (A) Quantification of bacterial cell survival under hypoxic conditions. (B) Number of colonies after a 10^{24} dilution of the wild-type (GV3101), pecS::Km, and CompecS after 72 h of incubation at 28°C. (C) Quantification of the siderophores produced after 72 h of incubation at 28°C. (D) Siderophore production by the wild-type and pecS::Km strains on a solid chrome azurol S (CAS) plate. The orange halo surrounding a bacterial colony indicates the amount of siderophores produced. The data represent the mean and SD from three biological replicates. The asterisks represent statistically significant differences, based on a Student's t test. ns, not significant; *, P , 0.05; **, P , 0.01.

difference in the quantity of exopolysaccharides produced by the wild-type and pecS::Km strains after 6, 14, and 24 h of culture (Fig. 4C), suggesting that PecS is not required to control EPS production. However, when pecS was expressed ectopically from a multicopy plasmid, increased EPS production was observed (after 24 h, the complementing plasmid was lost due to the absence of antibiotic pressure, as evidenced by failure to grow in the presence of the antibiotic, rationalizing why EPS production returned to the levels observed in the wild-type cells). This indicates that excess PecS may activate a key gene that is involved in EPS production or perhaps interferes with repression. It is conceivable that this elevated EPS production is related to the spreading phenotype of the CompecS strain (Fig. 4B).

pecS::Km grows poorly in microaerobic conditions. A deficiency in biofilm formation could be related to survival under conditions of limited oxygen, which is a condition that would characterize the interior of a biofilm community. Therefore, we investigated the survival rate of wild-type, pecS::Km, and complemented strains in a hypoxic environment. Cells were grown in Hungate tubes with a rubber stopper to ensure limited oxygen. Consistent with the reduced biofilm formation, the pecS::Km strain grew poorly in an environment of limited oxygen (Fig. 5A), as indicated by reduced formation of colonies (Fig. 5B). Both the wild-type and the complemented strains grew well under microaerobic conditions up to 72; however, the complemented strain showed a higher survival rate compared to that of the wild-type cells, particularly after 96 h, likely due to the overexpression of pecS from the multicopy plasmid (Fig. 5A). All of the strains showed reduced survival af-ter 120 h.

Iron limitation is a key environmental stress that plant pathogens must overcome during host infection (26). We used a Chrome Azurol S (CAS) assay to investigate the possible implication of PecS in siderophore production. In this assay, the chelation of iron by siderophores released from the bacteria causes release of orange CAS dye from a blue iron-containing CAS-complex. However, no difference in siderophore production was observed between the wild-type and pecS::Km strains, as evidenced by comparable orange halos

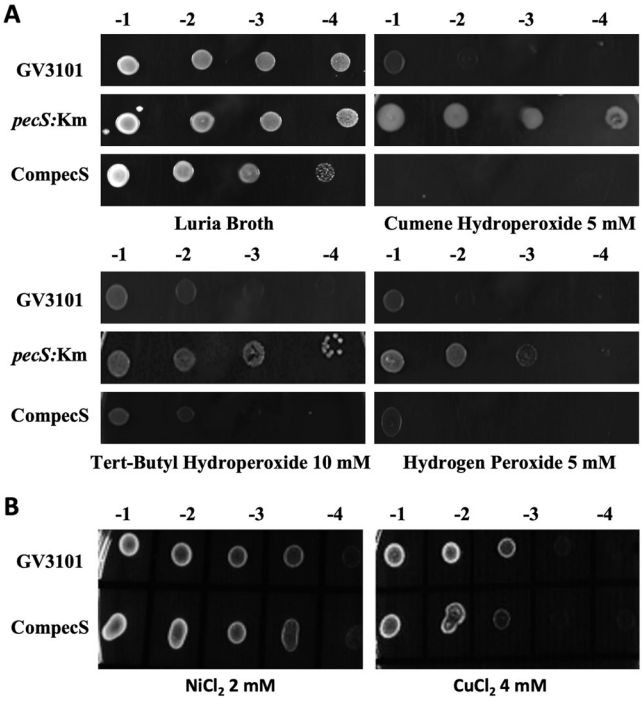

FIG 6 Oxidant and metal ion sensitivity of the GV3101, pecS::Km, and CompecS strains. (A) The wild-type (GV3101), pecS::Km, and complemented strains, spotted on LB plates without oxidant exposure (Luria Broth) or after exposure to the indicated oxidants. (B) Wild-type and pecS::Km strains spotted on LB plates after exposure to $NiCl_2$ and $CuCl_2$. Log-phase cells grown in LB were incubated for 30 min with the indicated concentrations of the oxidants or metal ions before 5 mL of serially log_{10}-diluted cells were spotted and grown on LB agar for 48 h. The experiment was performed in three biological replicates.

surrounding the bacterial colonies (Fig. 5C and D), suggesting that PecS does not contribute to the regulation of siderophore production.

PecS is implicated in the response of A. fabrum to oxidants but not to metal ions. The first line of defense of host plants against an invading pathogen is the release of reactive oxygen species (ROS); hence, a successful host colonization by bacterial spe-cies depends on their abilities to ward off host defenses (10). In D. dadantii, the PecS regulon was shown to include genes that are involved in the oxidative stress response, which is essential for infectivity and disease progression (5). We investigated the role of A. fabrum PecS in response to both organic and inorganic oxidants, and we found a reduced sensitivity of the pecS::Km strain to 5 mM cumene hydroperoxide, 10 mM tert-butyl hydroperoxide, and 5 mM hydrogen peroxide (Fig. 6A). In contrast, both wild-type and complemented strains exhibited sensitivity to these oxidants (Fig. 6A). This suggests that PecS contributes to oxidative stress responses in A. fabrum by maintaining genes that encode stress response factors in their repressed states until the bacteria encounter the inducing ligands for PecS in a host environment where ROS are produced.

Maintaining metal homeostasis is a critical task for the survival of bacteria (27). Since A. fabrum is a soilborne plant pathogen that is always exposed to metal ions in the soil, we investigated whether PecS is implicated in regulating responses to metal ions. No phenotypical difference was seen between the wild-type and pecS::Km strains, as both cells showed sensitivities to copper and nickel ions (Fig. 6B). The same outcome was observed when cobalt and zinc ions were used (data not shown).

AHL production is increased in the pecS::Km strain. Quorum sensing, which is a method of cell-to-cell communication, is involved in the horizontal transfer and vegetative replication of the Ti plasmid in Agrobacterium (28). Therefore, we investigated whether A. fabrum PecS is implicated in quorum sensing by measuring acyl homoserine lactone (AHL) concentrations in the growth medium, using a b-galactosidase-based biosensor strain that was derived from A. tumefaciens KYC55. No AHL was detectable in LB

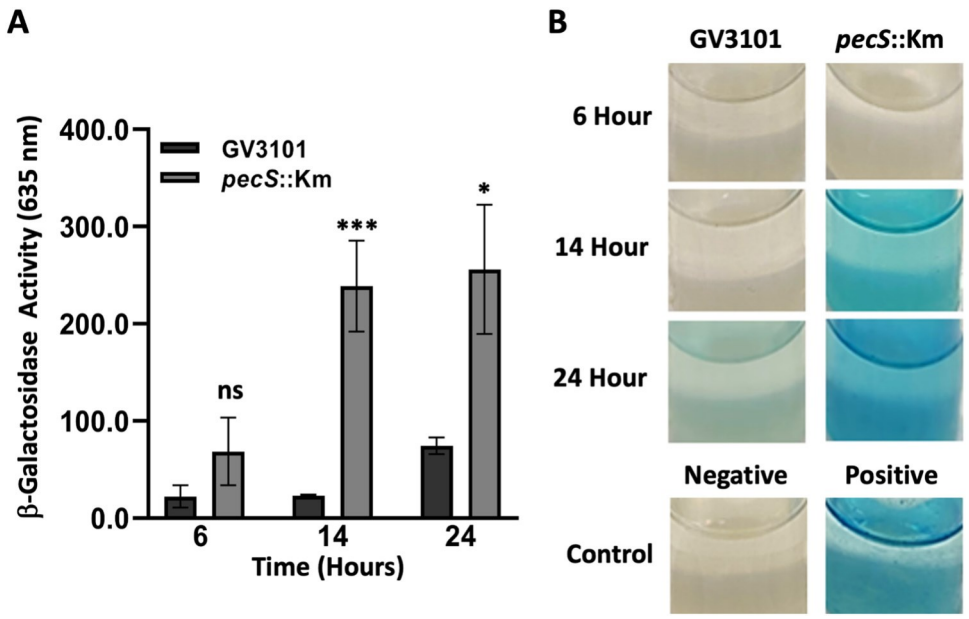

FIG 7 Quantitative acyl homoserine lactone (AHL) production. (A) AHL quantification from the culture supernatant of the wild-type and pecS::Km at the indicated time points. (B) Representative b-galactosidase assay from the culture supernatant of the wild-type and pecS::Km grown in LB broth. C8-HSL (10 mM) was added to the positive control, whereas no C8-HSL was added to the negative control. The asterisks represent statistically significant differences, based on a Student's t test. ns, not significant; *, P , 0.05; ***, P , 0.001.

medium from either the wild-type or the pecS::Km strains after 6 h of growth (Fig. 7A and B). However, a higher level of AHLs was observed in the pecS::Km strain, in both the exponential (14 h) and stationary (24 h) phases. These data suggest that AHL production is repressed by PecS.

The disruption of pecS results in differential responses to antibiotics. MarR proteins are often implicated in resistance to antibiotics (1). Therefore, we investigated the role of PecS in antibiotic resistance. The sensitivities of wild-type, pecS::Km, and complemented strains to the antibiotics ampicillin, streptomycin, and trimethoprim were assessed using a plate assay. The pecS::Km strain showed no sensitivity to 25 mg mL^{21} ampicillin, whereas sensitivity was shown by the wild-type and complemented strains (Fig. 8A). Conversely, both the wild-type and complemented strains were resistant to 25 mg mL^{21} trimethoprim and 200 mg mL^{21} streptomycin, whereas the pecS::Km strain showed sensitivity (Fig. 8B and C). No phenotypical difference was observed with nalidixic acid and tetracycline (data not shown).

Prediction of PecS target genes. For D. dadantii PecS, the inspection of a few PecS target gene promoters failed to yield a consensus sequence, and a selection strategy aimed at the identification of a preferred sequence resulted in a rather degenerate sequence, thereby suggesting an ability of PecS to interact with variable target sites (29). For the prediction of possible A. fabrum PecS target genes, we searched the genome using the regulatory sequence analysis tools (RSAT) genome-scale DNA-pattern algorithm (19). Using 59-TATCTTN$_6$AAGATA-39 as a query returned only the site in the pecS promoter, thereby raising the possibility that A. fabrum PecS may likewise be able to bind quite variable sequences. More degenerate query sequences did return candidate sites in gene promoters with some semblance to the cognate site in the pecS promoter. Of 12 such potential target genes, we selected two with the greatest similarity to the palindrome in the pecS promoter as well as some with apparent relevance to the identified phenotypes of the pecS::Km strain, regardless of whether or not a PecS site was predicted. The expression of these genes was determined in the WT, pecS::Km, and CompecS strains using RT-qPCR.

Two genes harboring a sequence with greater similarity to the PecS site in the pecS promoter were yggT and mltB. Atu2660 encodes YggT, which is a protein with predicted roles in osmotic shock tolerance, and the gene features a sequence with the greatest

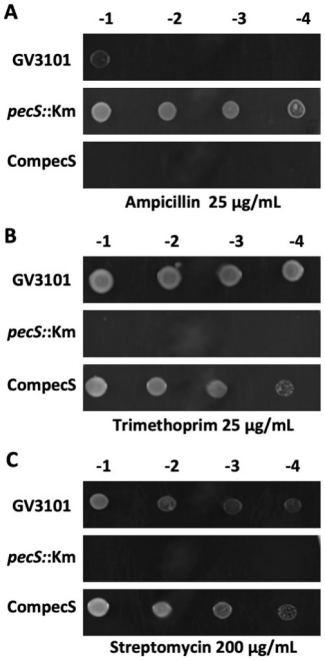

FIG 8 Sensitivity of GV3101, pecS::Km, and CompecS strains to antibiotics. (A) The wild-type, pecS::Km, and complemented strains on plates containing 25 mg mL^{21} ampicillin. (B) The wild-type, pecS::Km, and complemented strains on plates containing 25 mg mL^{21} trimethoprim. (C) The wild-type, pecS:: Km, and complemented strains on plates containing 200 mg mL^{21} streptomycin. 5 mL of log-phase cells that were grown in LB were serially log_{10}-diluted, spotted, and grown on LB agar containing the indicated antibiotics. The experiment was performed in three biological replicates.

similarity to the perfect palindrome in the pecS promoter (59-TgTCTTGcaacCAAGATA-39, where the lowercase letters differ). The expression of yggT was not significantly increased in pecS::Km cells (Fig. 9A). Atu3779 encodes a murein transglycosylase B (MltB), which has been reported to be required for induced resistance to ampicillin (predicted site, 59-TATCcctcCtgagAGATA-39) (30). The inactivation of pecS resulted in approximately threefold higher mltB expression, and complementation with pecS restored the repressed state (Fig. 9B). This suggests that PecS represses mltB, which is an observation that is consistent with the increased resistance of pecS::Km cells to ampicillin (Fig. 8A). The remaining candidate genes generally featured more divergent sites or a predicted function that was not obviously related to the phenotypes that were observed for the pecS::Km strain. No candidate sites were identified in the traI or traR genes, which are associated with quorum sensing, nor were oxidative stress response genes among the potential targets.

PecS does not mediate the expression of vir genes. The tumor formation in plants is initiated by the expression of A. fabrum vir genes that are encoded on the Ti plasmid, with the vir genes being activated by VirG in the presence of acetosyringone

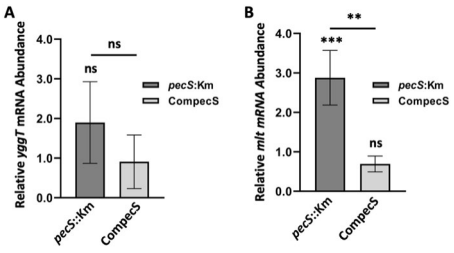

FIG 9 Differential expression of predicted PecS target genes. The relative abundance of transcript levels in pecS::Km and CompecS strains. The mRNA levels were measured by RT-qPCR, and the relative abundance was calculated, relative to wild-type cells, using the comparative C_T method. The asterisks represent statistically significant differences, compared to wild-type cells, unless indicated otherwise, based on a Student's t test. ns, not significant; *, P , 0.05; ***, P , 0.001.

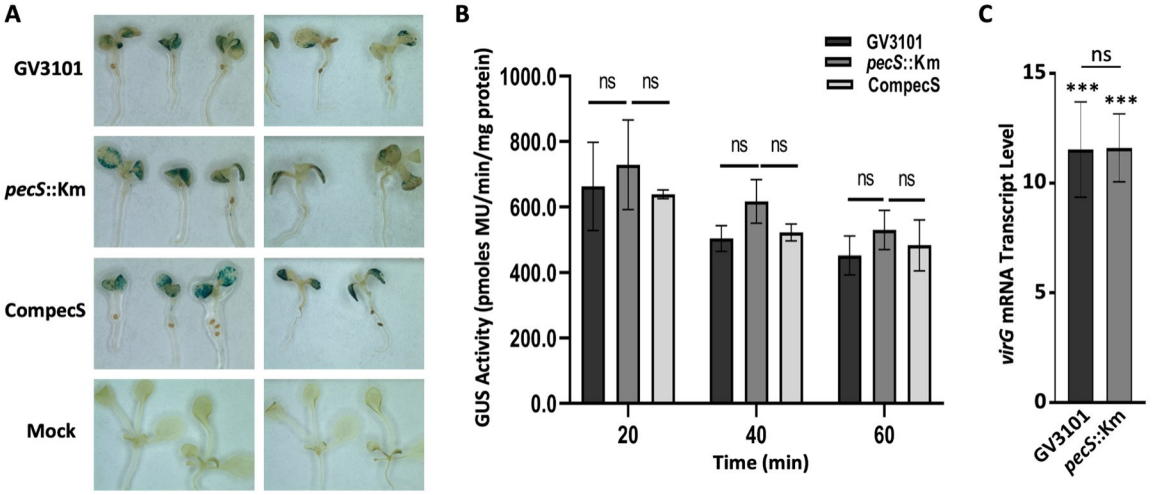

FIG 10 Transient transformation of Arabidopsis seedlings by Agrobacterium strains. (A) Transient GUS staining determined at 3 dpi. (B) Quantitative GUS activity. Four-day old Arabidopsis Col-0 seedlings were infected with the GV3101, pecS::Km, and CompecS strains. Seedlings grown in the same cocultivation medium without Agrobacterium infection are indicated as mock. (C) Relative change in virG transcript levels in wild-type and pecS::Km after growth in the presence of 200 mM acetosyringone, compared to cultures grown without acetysyringone. The asterisks reflect statistically significant virG induction by acetosyringone. The data represent the means from three biological replicates with the standard deviations. The asterisks represent statistically significant differences, based on a Student's t test. ns, not significant; ***, P , 0.001.

(13). To determine whether PecS is implicated in A. fabrum vir gene expression, we used a binary vector system in which a reporter gene encoding b-glucuronidase (GUS) is transferred from pCAMBIA 1105.1 to the host plant, thereby allowing the detection of the plant-based expression of GUS. Young Arabidopsis Col-0 seedlings were transi-ently transfected with wild-type, pecS::Km, and CompecS strains carrying pCAMBIA 1105.1. All infected seedlings showed GUS staining (Fig. 10A). Although some seed-lings showed more staining, this variable staining pattern was similar for all of the transfected seedlings (Fig. 10A). The mock plants did not show any GUS staining (Fig. 10A, bottom panel). The quantification of the GUS enzyme activity showed approxi-mately equal activity for seedlings transfected with either strain, suggesting that A. fabrum PecS does not directly regulate the expression of the vir genes. Consistent with this interpretation, we found comparable acetosyringone-mediated accumulation of virG mRNA in the wild-type and pecS::Km strains (Fig. 10C).

DISCUSSION

Differential regulation of pecS and pecM. While PecS was expected to repress both pecS and pecM based on the location of cognate sites within both pecS and pecM promoters (Fig. 1A), a single palindromic sequence makes up the binding site in the pecS promoter, whereas two PecS dimers simultaneously bind the pecM promoter (Fig. 1A). This difference in PecS occupancy may contribute to the differential induction of pecS and pecM genes on the addition of ligand. In the presence of 10 mM urate, com-parably increased pecS and pecM expression was observed (10). In contrast, lower con-centrations of xanthine more effectively enhanced pecS expression (Fig. 1E). Both ligands bind PecS with similar affinity (approximately 9 mM). However, differences in binding mode were reported in vitro; while urate substantially attenuates PecS binding to the pecS-pecM intergenic DNA, xanthine only effects a partial attenuation of DNA binding, which reaches a plateau at approximately 50% residual DNA binding (10). The latter is consistent with the observation that pecM remained repressed on the addition of xanthine. In addition, urate binds PecS with negative cooperativity, whereas xan-thine does not. Such negative cooperativity may result in the induction of PecS target genes only when cellular levels of urate reach a certain threshold. In contrast, the PecS regulon may be gradually induced as soon as xanthine levels increase. The physiological

reason for maintaining pecM in a repressed state in the presence of the ligand xanthine is unclear, but it might allow for the induction of the PecS regulon, with the exception of pecM. We speculate that this may be related to the (unknown) substrate(s) for PecM; if the PecS ligand is also a substrate for PecM, the efficient induction of pecM at a low ligand concentration may result in the premature extrusion of the inducing ligand. In D. dadantii, the substrate for PecM is the antioxidant indigoidine (31). The indigoidine biosynthetic genes are not conserved in A. fabrum, suggesting that A fabrum PecS transports different substrates.

PecS represses motility and chemotaxis. The motility of A. fabrum is mediated by flagella (21). Along with chemotaxis, motility is linked to biofilm formation and virulence, as bacteria in the rhizosphere move toward plant wounds and are attracted by plant exudates. Once at the plant surface, a transition from a motile to a sessile lifestyle occurs, which includes the repression of flagellar motility. This, in turn, promotes stable surface attachment and the establishment of a biofilm community (32). PecS appears to participate in the control of several of these events.

In A. fabrum, genes encoding structural components of the flagella as well as their assembly factors are encoded in a large gene cluster, and the majority of genes associated with chemotaxis are likewise encoded within a single gene cluster (22, 23). The increased motility (Fig. 3A and B) of the pecS::Km strain could be explained by the PecS-mediated repression of flagellar genes. Since the pecS disruption did not result in the increased expression of visN and visR (Fig. 3C and D), we deduce that PecS does not participate in the production of the regulators VisR and VisN, which have previ-ously been linked to the regulation of flagellar motility.

The rhizosphere is a complex environment that is enriched in compounds that are released from plant roots and from the microbial communities that inhabit this area (18). Since purine metabolites are present in the soil, the PecS ligands may lead to the differential expression of PecS target genes to enhance motility and chemotaxis, which are required for A. fabrum to reach wound sites.

The absence of PecS interferes with biofilm formation and microaerobic survival. Biofilm formation is a complex microbiological process that involves several stages of development, one of which is the adherence to surfaces (24). As noted above, this process includes the suppression of flagellar motility, which facilitates irreversible attachment. Initial surface contact may be made by flagella and followed by transient attachments by protein-rich adhesins and pili as well as ultimately irreversible attachment due to EPS production (22, 24). The pecS disruption could have compromised several of these stages, except perhaps EPS production (Fig. 4C), resulting in reduced biofilm formation (Fig. 4A and B). However, the expression of pecS from a multicopy plasmid resulted in elevated EPS production, suggesting that excess PecS does interfere with the proper regulation of the genes that are associated with EPS production.

Our data suggest that absence of the inducing ligands for PecS is conducive to biofilm formation. The biofilm matrix is notoriously protective, for instance, conferring tolerance to antimicrobials, thereby suggesting a reduced likelihood of exogenous urate and xanthine reaching cells that are embedded in the biofilm to act as inducing ligands for PecS. Therefore, sources of purines within the biofilm matrix are more likely limited to the lysis of dead cells, suggesting a reduced probability of the differential expression of the PecS regulon in this environment. We should also note that the starvation-induced stringent response in A. fabrum is associated with decreased cellular levels of urate and a concomitant reduction in pecS/pecM expression (33). This observation led to the inference that PecS is induced by exogenous ligands.

Within a tumor and within a biofilm community, a hypoxic gradient develops, thereby resulting in a hypoxic stress; under such circumstances, Agrobacterium may use denitrification as an alternative energy supply by using nitrate as the terminal electron acceptor in the electron transport chain (34). The disruption of pecS confers a disadvantage under microaerobic conditions (Fig. 5A and B), perhaps due to the misregulation of the genes that encode enzymes that are involved in the utilization or transport of alternative electron acceptors.

Oxidative stress responses are regulated by PecS. Successful host colonization by bacteria involves surviving the oxidative burst that is released by the host during infection (35). One of the mechanisms employed is the mobilization of xanthine dehydrogenase, which can use molecular oxygen as an electron acceptor while converting hypoxanthine to xanthine and xanthine to urate, thereby generating the superoxide anion (O_2^2) and H_2O_2 (17). Evidently, the absence of PecS, and, by extension, the presence of its inducing ligands, greatly enhances the ability of A. fabrum to survive oxidative stress (Fig. 6A). D. dadantii PecS regulates the indABC genes, which encode proteins that are required for the biosynthesis of the antioxidant indigoidine (5, 31). This raises the possibility that A. fabrum PecS might likewise regulate the production of an antioxidant, resulting in the observed survival of the pecS::Km strain in the presence of both organic and inorganic oxidants. Alternatively, the absence of PecS could have caused the upregulation of general stress response genes. The specific targets of PecS notwithstanding, our data indicate that PecS contributes to an inducible resistance to oxidants.

Quorum sensing. As bacterial cells proliferate, AHLs generally accumulate and interact with the AHL-responsive transcription factor (a LuxR homolog), which, in turn, activates QS-dependent genes, including luxI, which encodes the AHL synthase. In A. fabrum, the TraR/TraI QS system, which are the main LuxR/LuxI homologues, are encoded on the Ti plasmid (28). TraR-mediated gene regulation is key to Ti plasmid replication and conjugation and, in turn, for virulence. However, additional regulatory mechanisms pertain, and these ensure that Ti plasmid conjugation occurs only under conditions of high cell density and in the presence of crown gall-derived opines. The greater AHL production in pecS::Km cells during both the exponential and stationary phases (Fig. 7A and B) suggests that PecS is involved in the regulation of a specific aspect of this complex QS system. Since PecS is expected to encounter its ligands, namely, urate and xanthine, within the host environment, our data suggest that PecS contributes to the suppression of QS until an infection has been established.

PecS controls resistance to antibiotics. Bacteria may exhibit resistance to antibiotics on account of changes in membrane permeability or the enzymatic modification of the drugs. A. fabrum has a natural resistance to ampicillin due to the presence of the ampC-ampR operon, which is responsible for inducible b-lactamase production (30). At 25 mg mL^{21} ampicillin, A. fabrum exhibits a growth defect, compared to cells that are grown on LB plates without ampicillin (30). In comparison to cells grown without antibiotics (Fig. 8A), we confirmed the bactericidal effect of 25 mg mL^{21} ampicillin on wild-type and complemented strains after 48 h on solid medium, whereas the pecS::Km strain was resistant to this concentration (Fig. 8B). It was recently reported that absence of the cell wall remodeling enzyme MltB3 causes a hypersensitivity to ampicillin and a failure to induce resistance (30). The increased mltB expression in pecS::Km cells could explain the observed resistance to ampicillin. Conversely, the pecS::Km strain was sensitive to 25 mg mL^{21} trimethoprim and 200 mg mL^{21} streptomycin, whereas the WT and complemented strains were resistant (Fig. 8B and C). Resistance to streptomycin is typically due to its enzymatic inactivation (36), suggesting that sensitivity in the pecS::Km strain may be a result of the reduced expression of the corresponding genes. For trimethoprim, resistance is commonly achieved by altered membrane permeability or by the increased expression of its target, namely, dihydrofolate reductase, both of which could potentially be controlled by PecS.

Prediction of the PecS regulon. Early studies of genes encoding pectinolytic enzymes in D. dadantii (then, Erwinia chrysanthemi) revealed that several regulatory transcription factors are involved, aside from PecS. In addition, the affinity of D. dadan-tii PecS for certain regulatory sites is relatively low, and this led to the suggestion that cofactors might be required. There are also genes on which D. dadantii PecS appears to act as an anti-repressor by competing for overlapping binding sites (37). PecS-family transcription factors working in concert with other transcription factors was also shown for the Burkholderia thailandensis-encoded homolog MftR, for which both gene expression patterns and DNA binding to two specific sites with only modest similarity to the optimal sequence in the mftR promoter point to a requirement for cofactors (38, 39).

TABLE 1 Bacterial strains and plasmids

Strains	Description	Source
Escherichia coli		
RHO3	SM10(λpir) Dasd::FRT DaphA::FRT, Kan^R	48
Agrobacterium fabrum		
GV3101	Rif^R, Gen^R wild-type strain of C58C1 harboring pTiC58DT-DNA	49
pecS::Km	Rif^R, Gen^R, Km^R, GV3101 with disruption of pecS gene.	This study
CompecS	Rif^R, Gen^R, Km^R Chl^R, pecS::Km strain complemented with PecS, named CompecS.	This study
GV3101(pCAMBIA1105.1)	Rif^R, Gen^R, Spc^R and Hyg^R wild-type strain harboring plant expression vector.	This study
pecS::Km (pCAMBIA1105.1)	Rif^R, Gen^R, Km^R, Spc^R and Hyg^R pecS::Km strain harboring plant expression vector.	This study
CompecS (pCAMBIA1105.1)	Rif^R, Gen^R, Km^R, Chl^R, SPc^R and Hyg^R complemented strain harboring plant expression vector	This study
Plasmids		
pBBRBAD2	Expression vector for complementation. P_{BAD} promoter and araC gene cloned into pBBR1MCS-1, Chl^R	50
pB10	pecS gene cloned into pBBRBAD2, Chl^R	This study
pKNOCK-Km	Km^R, suicide vector for pecS disruption	51
pK20	pecS gene fragment cloned into pKNOCK-Km, Km^R	This study
pCAMBIA 1105.1	Spc^R and Hyg^R, pBR322 ori, GUS Plus. Plant expression vector for cloning and expression of GUS reporter gene in plants	Marker Gene Technologies

The distribution of the pecS-pecM gene pair is sporadic, suggesting its distribution by horizontal gene transfer (40). This implies the integration of PecS and PecM into existing regulatory circuits. Therefore, it is conceivable that PecS serves as a global regulator, acting in concert with locus-specific regulators to effect differential gene expression. An interaction with local regulators may lessen the requirement for highly conserved target sites (and confound a prediction of cognate sites), as was previously suggested for the function of D. dadantii PecS.

Physiological implications. Unlike other plant pathogens, A. fabrum does not aim to kill its plant host. Rather, it exploits the host to create a new ecological niche. The expression of vir genes and the transfer of T-DNA to the plant genome is key to plant transformation; these processes are not directed by PecS (Fig. 10). Instead, we propose that PecS functions at sequential steps leading up to the plant invasion to promote bacterial fitness. In the rhizosphere, where purine metabolites occur due to DNA and RNA turnover, PecS target genes may be induced, thereby facilitating the chemotactic motility and, in turn, the ability of the bacteria to reach plant wounds. As cells transition to a sessile lifestyle and form a biofilm community, we propose that access to the PecS ligands is attenuated, favoring biofilm formation and reduced motility. Once cells enter the plant host, they encounter ROS (in part produced by xanthine dehydrogenase), which is a process that leads to a marked accumulation of xanthine and urate (17). As a consequence, PecS target genes, including genes associated with oxidative stress responses, are induced along with genes linked to AHL production and the required propagation of the tumor-inducing plasmid. Taken together, our data therefore suggest that PecS mediates the fitness of A. fabrum during its transition from the rhizosphere to the host plant.

MATERIALS AND METHODS

Growth media and stock solutions. Tables 1 and 2 list all of the bacterial strains, plasmids, and oligonucleotides that were used. Unless otherwise stated, strain GV3101 (referred to as the wild-type) was grown at 28°C in Luria-Bertani medium supplemented with 50 mg mL^{21} of both rifampicin and gentamicin. The pecS disruption strain was grown similarly, but with the addition of 50 mg mL^{21} kanamycin, whereas the complemented strain was grown under the same conditions as was the pecS disruption strain, but with the addition of 35 mg mL^{-1} chloramphenicol. E. coli RHO3 was grown at 37°C in low salt LB containing 200 mg mL^{21} 2,6-diaminopimelic acid (DAP). An overnight culture was diluted 1:100 for subculture, unless indicated otherwise. For assays requiring urate and xanthine, cells were grown in 2 YT (yeast extract tryptone) medium, and both urate and xanthine were dissolved in 0.4 M NaOH and used at the indicated final concentrations.

The model plant Arabidopsis thaliana ecotype Columbia-0 (Col-0) was used for the plant infections. Seeds were sterilized in 50% bleach (vol/vol) containing 0.05% Tween 20 (vol/vol) for 5 min. After aspirating the bleach solution, 70% ethanol was used to wash the seeds. Ethanol was removed immediately, and the seeds were washed 5 times with sterile water. For germination, 50 seeds were transferred to

TABLE 2 Oligonucleotides

Primer sequence (59 to 39)	Purpose
pecS_KO_forward: TTGCCGCGGTCAGCACGTATCTTGG	Amplification of pecS fragment for disruption. SacII and SalI restriction enzyme sites underlined.
pecS_KO_reverse: ATCGTCGACTCGACGATGGCAAAGCCA	
pecS_com_forward: GTTATGCCTTGGTGATGAGCAAGAA	Confirmation of pecS disruption.
pecS_com_reverse: TTAGCAGCCCTTGCGCCCTGAGTG	
pecS_forward: GATATATCTAGAATGGACCACGTCGACCATATTC	Amplification of entire pecS coding sequence for complementation. XbaI and SacI restriction enzyme sites underlined.
pecS_reverse: ATATATGAGCTCCTATTCCTCGAAGTCCTGCAG	
P1Forward: TATTCCTCGAAGTCCTGCAGAA	Confirmation of complementation vector.
P1Reverse: TCTCTACTGTTTCTCCATACCCGT	
pecS_Ex_forward: CAGCGTCTTGATATCGCTGA	pecS gene expression.
pecS_Ex_reverse: CTCGAAGTCCTGCAGAAACC	
pecM_Ex_forward: ACCTCACCTTCACAGCTTGG	pecM gene expression.
pecM_Ex_reverse: CCCACGAAACCACAGAAGAT	
rpoA_Ex_forward: GAGCGTGGTTTCGGTCTTAC	rpoA gene expression.
rpoA_Ex_reverse: CATCTTGATGGCGATTTCCT	
virGF: GTTGGGAGCAACCGATTTTA	virG gene expression.
virGR: TTTCACCTCACTGCCCTCTT	
YggTF: GGGAGTTTCCTGGTGAATGT	yggT gene expression.
YggTR: TCGCCTGAGATGAAGGTAATG	
MltBF: TTCCCGCGTCAATCCTTATAC	mltB gene expression.
MltBR: GTCGGAGGCGTTGTATTTCT	
VisRF: CGACATTCTGCTCGACCATATC	visR gene expression.
VisRR: AGCTTGACCGCAATCTCTTC	
VisNF: GAACTCACCAACAGCCAGAA	visN gene expression.
VisNR: GTATTGCGTGAAATGCCAAGG	

5 mL 1/2 MS liquid medium (1/2 MS salt supplemented with 0.5% sucrose (wt/vol), pH 5.5 [pH adjusted to 5.7 with KOH, but pH 5.5 after autoclaving]) in a 40 mm diameter petri dish. Germination and growth took place in a growth room at 22°C under continuous light. The preinduction medium, namely, AB-MES, contained 17.2 mM K_2HPO_4, 8.3 mM NaH_2PO_4, 18.7 mM NH_4Cl, 2 mM KCl, 1.25 mM $MgSO_4$, 100 mM $CaCl_2$, 10 mM $FeSO_4$, 50 mM MES, 2% glucose (wt/vol) (pH 5.5) [37], while the cocultivation medium, namely, ABM-MES, contained 1/2 AB-MES, 1/4 MS, 0.25% sucrose (wt/vol) (pH 5.5).

Construction of the pecS disruption strain and complementation vector. To disrupt pecS, part of the pecS coding sequence was amplified from A. fabrum GV3101 genomic DNA using the pecS_KO_forward and pecS_KO_reverse primers that were flanked with SacII and SalI restriction sites. The PCR product was cloned into the pKNOCK-Km suicide vector, which was cut with the same restriction enzymes. The construct was then transformed into A. fabrum GV3101 via biparental conjugation using donor E. coli RHO3 cells in a donor:recipient ratio of 1:10. The cells were grown to an OD_{600} of approximately 0.8, and appropriate volumes of the donor and recipient cell cultures were separately washed twice to remove residual antibiotics. Each cell pellet was resuspended in a 50 mL cold NaCl solution (0.9 g L^{21}). Donor and recipient cells were mixed and incubated for 5 min at room temperature before spotting the mixture on a 0.2 mm membrane filter that was placed on an LB agar plate containing 200 mg mL^{21} DAP. Conjugation mixtures were incubated at 28°C for 16 h, after which the cells were collected and resuspended in 4 mL of 10 mM $MgSO_4$. The diluted cultures were spread on LB agar plates containing 50 mg mL^{21} rifampicin, 10 mg mL^{21} gentamicin, and 50 mg mL^{21} kanamycin. A. fabrum colonies exhibiting kanamycin resistance were screened by colony PCR, using the pecS_com_forward and pecS_com_reverse primers to verify the disruption of the pecS gene. The strain was named pecS::Km to reflect the presence of the kanamycin resistance cassette.

A complementation vector was constructed by amplifying the entire pecS coding sequence from A. fabrum GV3101 genomic DNA, using pecS_forward and pecS_reverse primers that contained XbaI and SacI restriction sites. The PCR product was then cloned into the expression vector pBBRBAD2, behind the arabinose inducible P_{BAD} promoter. The construct was confirmed by DNA sequencing and trans-formed into the A. fabrum pecS::Km strain via biparental conjugation, as described above. The comple-mentation of A. fabrum pecS::Km cells was verified by chloramphenicol resistance and colony PCR, using the P1Forward and P1Reverse primers.

Prediction of PecS target genes. The A. fabrum C58 genomic DNA sequence was queried using the RSAT genome-scale DNA-pattern algorithm (41). Both strands were searched, allowing for overlapping matches, using as query patterns sequences representing variations on the perfect palindrome 39-TATCTTGACGTCAAGATA-59 in the pecS promoter (Fig. 1A) (10). Sequences upstream of annotated genes were searched, allowing for overlap with upstream ORFs. The sequences that were used as queries allowed the central 6 bp to vary, yielding 39-TATCTTN$_6$AAGATA-59 or 39-TATCTCN$_6$GAGATA-59 (based on the somewhat degenerate binding sites in the pecM promoter) while allowing one additional base pair to differ from the query pattern. More degenerate query sequences yielded too many hits to serve as a meaningful prediction. A potential role for PecS in controlling candidate gene expression was determined by RT-qPCR, as described below.

In vivo gene expression analysis using RT-qPCR. LB medium (5 mL) was inoculated with a single bacterial colony, and the culture was grown for 48 h at 28°C. The cells were diluted 1:100 and grown at

28°C. The cells were harvested and washed twice with cold diethyl pyrocarbonate (DEPC) treated-water, re-pelleted, and stored overnight at 280°C. Total RNA was isolated using a Monarch Total RNA Mini Prep Kit (New England BioLabs). Turbo DNase (Thermo Fisher Scientific) was used to remove DNA contamination, and the absence of DNA was verified using PCR. NanoDrop and electrophoresis on 1.2% agarose gels were used to verify the integrity and quality of the RNA. The Luna Universal One Step RT-qPCR Kit (New England BioLabs) was used for quantifying the mRNA transcripts. In a 20 mL reaction mixture, gene-specific forward and reverse primers were added together with the isolated RNA and Luna univer-sal master mix to perform qPCR on a QuantStudio 6 real-time PCR system (Applied Biosystems). For the determination of the effects of urate and xanthine on the transcript levels, the 48 h culture was diluted 1:100 with 2 YT medium that contained 50 mg mL^{21} of both rifampicin and gentamicin. At an OD_{600} of approximately 0.4, various concentrations of urate or xanthine (dissolved in 0.4 M NaOH) were added, and cultures were grown for 45 min. The control culture was supplemented with an equal volume of 0.4 M NaOH. For the analysis of the virG expression, the 48 h culture was diluted in AB-MES medium without or with 200 mM acetosyringone and grown to an OD_{600} of approximately 0.8. The 2^{2DCT} method was used to estimate the transcript levels from three biological replicates, using rpoA as the reference gene (rpoA was reported to be constitutively expressed in A. fabrum [42]).

Biofilm formation. Cultures were grown for 48 h in LB medium. Fresh LB medium (1 mL) in polystyrene tubes was inoculated with 100 mL of culture that had previously been diluted to an OD_{600} of approximately 0.6. The culture was incubated at 28°C without shaking. After incubation for 24 h, the cultures were decanted, and the tubes were rinsed twice to remove unattached cells. To perform crystal violet staining, 1 mL 0.1% solution of crystal violet in water was added to the tubes, which were left at room temperature for 15 min, after which the crystal violet solution was decanted. The tubes were left to dry for several hours. A solution of acetic acid (30% in water) was used to solubilize the crystal violet, and the absorbance of the solubilized crystal violet was measured at 550 nm. The acetic acid solution was used as the blank. The data represent the means from three biological replicates.

AHL quantification. A. fabrum strains were analyzed for the production of N-acyl-homoserine lactones (AHLs) by using the b-galactosidase-based biosensor strain A. tumefaciens KYC55(pJZ372)(pJZ384)(pJZ410) (43). This strain responds to the presence of AHLs in the culture medium by expressing lacZ. The quantification of AHLs from the culture supernatant was performed as described (44). Agrobacterium strains were grown in LB broth at 37°C without antibiotics. The culture supernatant was collected by centrifugation at the indicated times and passed through a 0.22 mM filter. An equal amount of KYC55 cells (5 10^7 CFU/mL in a volume of 5 microL) were added to culture tubes containing 5 mL AT broth supplemented with 40 mg/mL X-gal. Tubes were incubated at 28°C for 6, 14, and 24 h. The control tubes were supplemented with 0 or 10 mM N-octanoyl-L-homoserine lactone (C8-HSL; Sigma-Aldrich). The development of blue color was read at 635 nm. The absorbance of the negative control was subtracted from the absorbance of each sample. The data were normalized to OD_{600} and are presented as the mean 6 SD from three replicates.

Exopolysaccharide production. The quantitative levels of secreted exopolysaccharides were compared between strains. Supernatants were collected from strains grown for 6, 14, and 24 h without antibiotics and were diluted to an OD_{600} of approximately 0.6. 50 mL of the supernatants were pipetted into tubes and then made up to 1 mL with 950 mL of distilled water. 4 mL of the anthrone reagent (200 mg dissolved in 100 mL conc. H_2SO_4) were added to each tube and vortexed. The tubes were placed in a boiling water bath for 10 min, after which they were cooled to room temperature. The absorbance of the solutions was measured at 620 nm. Distilled water was used as a blank. The experiments were performed at least three times.

Anaerobiosis. To compare the survival of the wild-type, pecS::Km, and complemented strains under conditions of oxygen limitation, the 48 h cultures were diluted to an OD_{600} of approximately 2.0, and 1 mL of cells was collected by centrifugation, washed twice with LB medium to remove any residual antibiotics, and resuspended in 1 mL LB. Hungate tubes were used to ensure gas-tight closures that prevented the permeation of air into the tubes. A culture volume of 150 mL was transferred into Hungate tubes filled with 15 mL LB (which left little headspace), and the tubes were incubated at 28°C. At each time point, the samples were withdrawn through the rubber septum, the OD_{600} value was measured, and 20 mL of cells were serially diluted and plated on LB agar plates. At 72, 96, and 120 h, the colonies were counted, corrected for dilution, and normalized to OD_{600}. The survival is presented, relative to the viability of cells at time zero from three replicates.

Siderophore production. Siderophore production was assessed using a Chrome Azurol S (CAS) assay as previously described (45). Bacterial strains were grown for 48 h at 28°C and diluted to an OD_{600} of approximately 0.6. 5 mL cultures were spotted at the centers of the blue CAS agar plates and incu-bated at 28°C for 3 days. The diameters of the orange color that formed were then measured. The data are presented as the mean 6 SD from three experiments.

Motility and chemotactic assays. The swimming phenotypes were determined using LB agar plates containing 0.3% agar. The swim plates were inoculated with 5 mL of overnight cultures (diluted to an OD_{600} of approximately 0.6) that were spotted at the center of the plate. The plates were incubated at 28°C, and the diameters of the colonies were measured after 48 h of incubation.

The chemotactic responses to sucrose, onion explant, and acetosyringone were assessed using AT minimal medium swim plates that contained 0.3% agar. The cultures were grown for 48 h at 28°C and diluted to an OD_{600} of approximately 0.6. For the response to sucrose, 20% sucrose was added to the AT minimal medium instead of glucose, and 5 mL of diluted cultures were spotted at the middle of the plates. The plates were covered and left at room temperature for 1 h to allow the inoculum to soak into the medium, and the plates were then incubated at room temperature for 48 h in a sealed container with a small, uncovered beaker of saturated potassium sulfate added to maintain humidity (46). The

diameters of the colonies were measured. For the response to explant, chopped onion was placed at the edge of the plates. Five mL of diluted cultures were spotted at the middles of the plates, and the ino-culated plates were treated as described for the response to sucrose. The response to acetosyringone was assessed by placing filter paper soaked in 0.2 M acetosyringone at the edge of the plate. The ratio (T/A) of the distance toward the explant over the distance away from the explant was measured. The data represent three replicates.

Sensitivity assays. The sensitivity to antibiotics, oxidants, and metals were assessed using solid agar plate assays. The cells were grown for about 48 h before a 1:100 dilution using LB medium. The diluted cultures were further grown at 28°C to an OD_{600} of approximately 0.6. For the susceptibility to antibiot-ics, 5 mL cell cultures were spotted on LB agar plates that contained the appropriate antibiotic (ampicil-lin, streptomycin, or trimethoprim). To determine the response to oxidants, 1 mL of each culture was incubated with either 5 mM H_2O_2, 5 mM cumene hydroperoxide, or 10 mM tert-butyl hydroperoxide for 30 min at 28°C before spotting on LB agar plates. To determine the response to metals, 1 mL of each cul-ture was incubated with the appropriate metal ion concentrations for 30 min at 28°C before plate spot-ting. For all of the sensitivity assays, the broth microdilution method was used to spot 5 mL of serially log_{10}-diluted bacterial cells onto the appropriate agar plates, which were then incubated at 28°C for 48 h before analysis. The data represent three replicates.

Infection of Arabidopsis seedlings with Agrobacterium strains and vir gene expression. Infection of Arabidopsis seedlings was done according to the Agrobacterium-mediated enhanced seedling transformation (AGROBEST) method (47). A. fabrum from an 280°C glycerol stock was freshly streaked onto an LB agar plate for 2 days of incubation at 28°C. A single colony was used to inoculate 5 mL of LB that contained the appropriate antibiotics for shaking (220 rpm) at 28°C for 20 to 24 h. For the preinduction of A. fabrum vir gene expression, the cells were pelleted and resuspended to an OD_{600} of approximately 0.2 in AB-MES without antibiotics, and they were then shaken (220 rpm) at 28°C for 16 h. The cells were then pelleted and resuspended in ABM-MS cocultivation liquid medium to an OD_{600} of approximately 0.02. Three-day-old seedlings were treated with 10 mM dexamethasone (DEX) for 1 day before infection for 3 days with 5 mL of freshly prepared A. fabrum cells. The removal of Agrobacterium cells after the chosen infection time was done by replacing the cocultivation medium with 5 mL of freshly prepared, full-strength MS medium that contained 100 mM of timentin to inhibit bacterial growth and then incubating the seedlings for 3 additional days before analysis. Unless indicated, 50 seedlings grown in each plate were infected, and 3 biological repeats were performed.

For the b-glucuronidase (GUS) assay, 15 seedlings were stained with 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) at 37°C for 24 h. Another 15 seedlings were homogenized and assayed for GUS activity with the GUS substrate 4-methylumbelliferyl-b-D-glucuronide (MUG), which is converted to a fluorescent product 4-methylumbelliferone (MU), as described by the manufacturer (Sigma-Aldrich). The fluorescence at various time points was determined using a 96 microtiter plate reader (356 nm excitation, 455 nm emission with a 620 nm filter), and the specific fluorescence intensity was based on the 4-MU standard curve that was obtained from the same microtiter plate. The relative GUS activity was obtained as described by the manufacturer, with the individual fluorescence signals being normalized to the amount of protein (determined using a Bradford assay) after the subtraction of the background fluorescence that was detected in the negative control.

Statistical analyses. The data are presented as the mean 6 standard deviation (SD). Each experiment was repeated three times with three independent biological replicates. The graphs were produced by using the GraphPad Prism 9.0 software package. Statistical significance was calculated using unpaired Student's t tests. Symbols: ns, not significant; *, P , 0.05; **, P , 0.01; ***, P , 0.001.

ACKNOWLEDGMENTS

We thank G. Pettis for the plasmid pBBRBAD2. This work was supported by the National Science Foundation (MCB-1714219 to A.G.).

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