www.nature.com/ismej

ISME

ARTICLE

Check for updates

The ColR/S two-component system is a conserved determinant of host association across *Pseudomonas* species

Christina L. Wiesmann[1,4], Yue Zhang[1,4], Morgan Alford[1,2], Corri D. Hamilton[1], Manisha Dosanjh[1], David Thoms[1], Melanie Dostert[1,2], Andrew Wilson[1], Daniel Pletzer[2,3], Robert E. W. Hancock[1,2] and Cara H. Haney[1]✉

Members of the bacterial genus *Pseudomonas* form mutualistic, commensal, and pathogenic associations with diverse hosts. The prevalence of host association across the genus suggests that symbiosis may be a conserved ancestral trait and that distinct symbiotic lifestyles may be more recently evolved. Here we show that the ColR/S two-component system, part of the *Pseudomonas* core genome, is functionally conserved between *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*. Using plant rhizosphere colonization and virulence in a murine abscess model, we show that *colR* is required for commensalism with plants and virulence in animals. Comparative transcriptomics revealed that the ColR regulon has diverged between *P. aeruginosa* and *P. fluorescens* and deleting components of the ColR regulon revealed strain-specific, but not host-specific, requirements for ColR-dependent genes. Collectively, our results suggest that ColR/S allows *Pseudomonas* to sense and respond to a host, but that the ColR-regulon has diverged between *Pseudomonas* strains with distinct lifestyles. This suggests that conservation of two-component systems, coupled with life-style dependent diversification of the regulon, may play a role in host association and lifestyle transitions.

The ISME Journal (2023) 17:286–296; https://doi.org/10.1038/s41396-022-01343-3

INTRODUCTION

Plants and animals are colonized by symbiotic bacteria. Symbionts include mutualists that help with nutrient uptake or pathogen protection, commensals that live in association with hosts without causing harm or benefit, and pathogens that cause disease. Transitions between symbiotic lifestyles [1, 2] and even hosts [3] can occur over relatively short evolutionary distances. While mechanisms underlying bacterial virulence and mutualism are widely studied, relatively little research has focused on the mechanisms by which bacteria initiate symbiotic host association and if these mechanisms are conserved across closely related pathogenic and commensal bacteria.

Both plants and animals have functionally similar receptors and signaling pathways that allow them to sense and respond to the presence of bacteria [4]. Accordingly, *Pseudomonas aeruginosa* makes use of the same virulence factors to infect both plants and animals [5, 6], suggesting that *P. aeruginosa* targets similar cellular processes across hosts. Consistently, the nitrogen-fixing plant mutualist *Sinorhizobium meliloti* has genes required for mutualism that are homologous to virulence factors in the animal pathogen *Brucella abortus* and the plant pathogen *Agrobacterium tumefaciens* [3]. Regardless of symbiotic lifestyle, host-associated bacteria must be able to colonize their host. As a result, we hypothesized that genes required for host colonization may be shared by pathogens, commensals, and mutualists.

The bacterial genus *Pseudomonas* includes plant commensals and opportunistic animal and plant pathogens [7–9] and thus provides a model genus to study conserved symbiotic mechanisms. We hypothesize that the ability to be symbiotic is ancestral within the genus *Pseudomonas*, and thus should be encoded by components of the core genome, with further evolution of species or strains leading to distinct lifestyles. Indeed, the *Pseudomonas* accessory genome (genes not conserved across the majority of *Pseudomonas* strains) has previously been shown to encode virulence factors [1, 10] while colonization factors may include components of the core genome [11].

Two-component systems are common signal-transduction cascades in bacteria that sense environmental cues, including the presence of a host, and cause altered gene expression. Orthologous two-component systems in *Brucella abortus* (BvrR/S), *Sinorhizobium meliloti* (ExoS/ChvI) and *Agrobacterium tumefaciens* (VirA/VirG), are required for virulence in animals and mutualism or virulence in plants [12–15]. The ColR/S two-component system is required for colonization of the tomato rhizosphere by *Pseudomonas fluorescens* WCS365 [16, 17], and ColS is required for *Pseudomonas aeruginosa* TBCF10839 virulence during infection of the nematode *Caenorhabditis elegans* [18]. As different species spanning the *Pseudomonas* genus can be host associated [19], we hypothesized that distantly related *Pseudomonas* species may use conserved two-component signaling pathways to sense and adapt to diverse host environments.

Because ColR/S has been identified in phylogenetically diverse *Pseudomonas* spp. as required for rhizosphere colonization [16, 20], or virulence in *C. elegans* [18], we tested whether ColR/S, and its

[1]Department of Microbiology and Immunology, The University of British Columbia, Vancouver, BC V6T 1Z3, Canada. [2]2259 Lower Mall Research Station, The University of British Columbia, Vancouver, BC V6T 1Z4, Canada. [3]Department of Microbiology and Immunology, University of Otago, 720 Cumberland St., 9054 Dunedin, New Zealand. [4]These authors contributed equally: Christina L. Wiesmann, Yue Zhang. ✉email: cara.haney@ubc.ca

Received: 11 August 2022 Revised: 7 November 2022 Accepted: 9 November 2022
Published online: 24 November 2022

regulon, are broadly conserved host-association factors across diverse *Pseudomonas* spp. and diverse hosts. The genes encoding for ColR/S are components of the core *Pseudomonas* genome, and previously identified components of the ColR regulon are broadly conserved across the genus [11]. ColS has been proposed to bind iron or zinc through a conserved ExxE motif [21]; as bacteria are known to colonize host environments with low pH [22, 23], and metals have higher solubility at low pH, this suggests ColS has the potential to sense diverse host environments. Collectively this indicates that identifying the genes and mechanisms by which ColR/S regulates host association may reveal conserved mechanisms that predispose members of the genus *Pseudomonas* for symbiosis.

To test the role of ColR/S in association across hosts, we used *P. fluorescens* WCS365, *P. aeruginosa* PAO1, and *P. aeruginosa* LESB58 as model strains to study mechanisms of plant and animal host association across the genus *Pseudomonas*. *P. fluorescens* WCS365 is a beneficial plant growth-promoting strain [24]. *P. aeruginosa* PAO1 is a human and plant opportunistic pathogen [25]. It can efficiently colonize the plant rhizosphere, but causes disease in plants including Arabidopsis, sweet basil, canola, and poplar trees [26–28]. *P. aeruginosa* PAO1 also quickly disseminates into the tissue of mice to cause infection, causing death of most mice within three days, making it an non-ideal strain for studying prolonged association with a murine host [29]. *P. aeruginosa* LESB58 was isolated from a cystic fibrosis patient and causes chronic infection in a subcutaneous murine infection model, making it an ideal strain for studying host association over many days [29]. We used growth of *P. aeruginosa* PAO1 and *P. fluorescens* WCS365 in an Arabidopsis rhizosphere model [30] to identify conserved or divergent ColR-dependent genes required for rhizosphere colonization. We then used both *P. aeruginosa* PAO1 and LESB58 in a murine subcutaneous abscess model to test if the genes required for rhizosphere colonization are also required for virulence in a murine model [29]. Collectively, use of these strains and hosts allows us to test whether ColR/S is broadly required for host association.

We generated deletions of *colR* in *P. aeruginosa* and *P. fluorescens* and found that ColR is required for rhizosphere colonization in both *P. fluorescens* and *P. aeruginosa*, and abscess formation by *P. aeruginosa*, indicating that ColR is required for host association across diverse strains and hosts. Using comparative transcriptomics, we found that ColR-dependent genes have largely diverged between *Pseudomonas* strains. We propose that ColR/S allows *Pseudomonas* to sense and respond to the presence of a host, but the ColR-regulon has diverged between *Pseudomonas* strains with distinct lifestyles.

RESULTS

The ColR response regulator is conserved and required for rhizosphere colonization across *Pseudomonas* species

To assess the degree of functional conservation of the ColR response regulator across the genus *Pseudomonas*, we used the model strains *P. fluorescens* WCS365, *P. aeruginosa* PAO1, and *P. aeruginosa* LESB58 to study common mechanisms required for host association. An alignment of the ColR and ColS sequences from more than 500 orthologs across the genus *Pseudomonas* shows a high degree of similarity across the genus with conserved predicted DNA- and zinc-binding domains, respectively (Supplementary Fig. 1). *P. aeruginosa* PAO1 and *P. fluorescens* WCS365 ColR and ColS are 88% and 61% identical at the amino acid level, respectively, suggesting that ColR and ColS are highly conserved between these two species (Supplementary Fig. 1).

To test the conservation of the requirement of *colR* in rhizosphere colonization, we deleted *colR* in *P. fluorescens* WCS365 and in *P. aeruginosa* PAO1. We found that consistent with disruption of *colS* being required for potato root tip colonization [16], deletion of *colR* leads to a seven-fold decrease in growth of WCS365 (Fig. 1A) and a ten-fold decrease in rhizosphere growth of PAO1 (Fig. 1B) when inoculated into the rhizosphere of hydroponically grown Arabidopsis seedlings. In contrast, *colR* mutants grew at similar rates as wild-type bacteria when grown in M9 minimal media or MS media with succinate as a sole carbon source (Supplementary Fig. S2) indicating that the Δ*colR* mutants do not have a general growth defect. Microscopy with *P. aeruginosa* PAO1 expressing GFP revealed lower abundance of the Δ*colR* mutant in the rhizosphere (Fig. 1C). Complementation of *colR* deletion mutants with the copies of the *colR* from their respective strains expressed under the *colR* native promoter restored rhizosphere colonization of the *colR* deletion mutants to wild-type levels or higher (Fig. 1A, B). The complemented WCS365 and PAO1 *colR* mutants had significant growth defects in minimal media (Supplementary Fig. S2) suggesting that expression of *colR* from its native promoter on a plasmid might result in overexpression and/or altered phosphorylation, which might be detrimental to growth in vitro but not in vivo. Because of the high degree of conservation of ColR across species, we tested if *P. aeruginosa* PAO1 *colR* could complement a *P. fluorescens* WCS365 Δ*colR* mutant in vivo. We found that PAO1 *colR* expression from a plasmid is able to complement the WCS365 Δ*colR* deletion and rescue rhizosphere growth (Fig. 1A). Collectively these data indicate that *colR* is a conserved rhizophore colonization factor across diverse *Pseudomonas*.

Because previous research has shown that virulence mechanisms are conserved across *Pseudomonas aeruginosa* infecting plants and animals [6, 31], we also tested if *colR* was required for virulence in a murine subcutaneous abscess model [29]. We deleted *colR* in the Liverpool Epidemic Strain *P. aeruginosa* LESB58 (Methods; Supplementary Dataset S1) and found that the *colR* mutant formed a significantly smaller abscess than wild-type LESB58 (54% reduction in size; 90 ± 9 mm for wild-type versus 48.6 ± 3 mM for the *ΔcolR* mutant), indicating that *colR* was required for virulence (Fig. 1E, F). In contrast to Δ*colR* mutants in the rhizosphere, the LESB58 *ΔcolR* mutant grew to similar levels as wild-type bacteria in an abscess (Fig. 1D) suggesting that *colR* in LESB58 may primarily contribute to virulence rather than growth in the abscess model. We also tested the more acutely infectious PAO1 *ΔcolR* mutant for abscess formation and found that the *ΔcolR* mutant did not have a pronounced virulence defect (Supplementary Fig. S3). Collectively these data indicate that ColR plays a role in host association through colonization or establishment of chronic infection in both *P. aeruginosa* and *P. fluorescens*.

The ColR regulons are highly divergent across *Pseudomonas* strains

The requirement of *colR* in symbiosis (commensalism or virulence) in *P. fluorescens* and *P. aeruginosa* led us to hypothesize that *colR* regulates an overlapping set of genes in these two species. As these two strains have distinct host-association lifestyles (beneficial rhizosphere bacterium vs. opportunistic human and plant pathogen), we hypothesized that *colR* may regulate genes required for host association rather than mutualism or virulence. To identify ColR-dependent genes in both *P. fluorescens* WCS365 and in *P. aeruginosa* PAO1, we performed RNA-Seq on wild-type bacteria and Δ*colR* mutants in the rhizosphere. We grew both PAO1 and WCS365 in the Arabidopsis rhizosphere and in M9 minimal media with 20 mM L-glutamine (L-gln) (*P. aeruginosa* PAO1) or 30 mM sodium succinate (*P. fluorescens* WCS365) and isolated RNA 6 h post-inoculation to identify rhizosphere-specific *colR*-dependent and independent responses. The sequencing reads from three biological replicates were mapped to the PAO1 or WCS365 transcriptomes. As previously only a draft genome of WCS365 was available, a complete WCS365 genome assembly was generated for use in this study (Methods).

To determine how divergent the transcriptomes were between wild-type bacteria and the *colR* mutants of the same strain, and between the rhizosphere and M9 minimal media, we performed principal component analysis (PCA) with the RNA-Seq data. We found that the majority of the differences in gene expression within a single strain (94–97%) were due to growth condition (media vs. rhizosphere) and only a small (1–2%) amount of variance in gene expression was due to bacterial genotype (wild-type vs. Δ*colR*) (Fig. 2A). This suggests that *Pseudomonas* undergo major transcriptional reprogramming in the rhizosphere, but that only a subset of these changes are ColR-dependent.

Because only a small portion of differences in transcription in the rhizosphere were ColR-dependent, we hypothesized that failure to induce expression of these genes may explain the requirement of ColR in rhizosphere colonization. We identified genes that were specifically upregulated in the rhizosphere in wild-type relative to a Δ*colR* mutant in both WCS365 and in PAO1 (Dataset S2; Methods). We identified 50 and 42 positively ColR-regulated genes with higher expression in the rhizosphere in wild-type than in the *colR* mutant in WCS365 and in PAO1, respectively (Fig. 2B, C). These data indicate that ColR may directly or indirectly upregulate a limited set of genes during rhizosphere colonization.

To determine if the ColR regulon is conserved between *Pseudomonas* spp. WCS365 and PAO1, we used the RNA-Seq data described above to perform comparative transcriptomics to identify orthologous genes present in the ColR regulons of PAO1 and WCS365 [1]. We identified only four genes that were ColR regulated in both PAO1 and WCS365 (Fig. 2B–D; Supplementary Dataset S2). These include *eptA* and *eptC* (which encode phosphoethanolamine transferases), *tpbA* (which encodes a tyrosine phosphatase), and *warA* (which encodes a methyltransferase) (Fig. 2B–D; Cluster 1). Of these, expression of *warA* was previously shown to be ColR-regulated in *P. putida* [32]. We found that 26 ColR-dependent genes in *P. fluorescens* WCS365 and 21 ColR-dependent genes in *P. aeruginosa* PAO1 had a ColR-independent ortholog in the other strain (Fig. 2B, C; Cluster 2 and 3, and Supplementary Fig. S4), indicating these genes have divergent regulation. The remaining genes in the ColR regulon encoded genes unique to PAO1 or WCS365; 24 ColR-dependent genes in WCS365 and 16 genes in PAO1 did not have any orthologs in the other strain (Fig. 2B–D; Cluster 4 and 5) and are not highly conserved across the genus *Pseudomonas* (Fig. 2D). Despite ColR serving a similar function in rhizosphere colonization by *P. aeruginosa* PAO1 and *P. fluorescens* WCS365, these results suggest the ColR regulon has largely diverged between PAO1 and WCS365.

Deleting components of the ColR regulon revealed strain-specific, but not host-specific, requirements for ColR-dependent genes

We reasoned that orthologous ColR-regulated genes shared in both *P. fluorescens* WCS365 and *P. aeruginosa* PAO1 might be part of a conserved ColR regulon required for host association. We tested if *eptA*, *tpbA*, and *warA*, which are ColR-dependent in both *P. fluorescens* WCS365 and *P. aeruginosa* PAO1, were required for rhizosphere colonization in both strains. We used either transposon insertion mutants (PAO1 *tpbA*::Tn5 and PAO1 *warA*::Tn5 [33]),

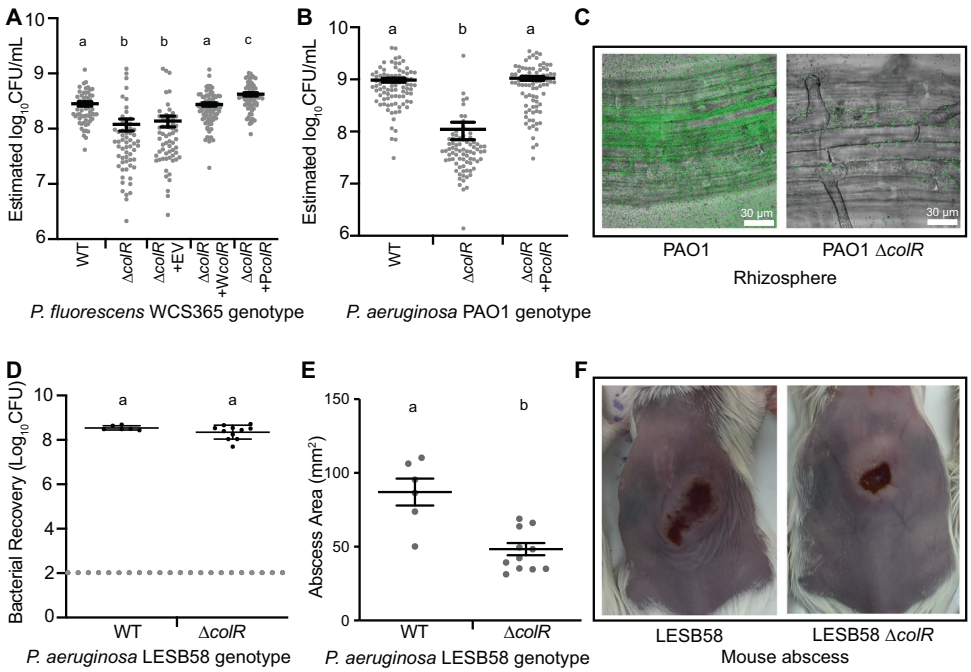

Fig. 1 ColR is a conserved host association factor across *Pseudomonas* spp. in plant and murine hosts. A To test if *colR* is necessary for rhizosphere colonization, seedlings growing hydroponically in 48-well plates were inoculated with GFP-expressing *P. fluorescens* WCS365, WCS365 Δ*colR*, WCS365 Δ*colR* containing an empty vector (EV), a vector expressing WCS365 *colR* under its native promoter (W*colR*), a vector expressing PAO1 *colR* under its native promoter (P*colR*) or (**B**) *P. aeruginosa* PAO1, PAO1 Δ*colR*, or PAO1 Δ*colR* expressing *colR* under its native promoter (P*colR*). **A**, **B** GFP fluorescence values in each plant-containing well were measured 5 days post-inoculation and converted to OD_{600} using a standard curve. Each data point represents the estimated CFU values from a single well containing a single plant. Experiments were repeated three independent times with 10-30 plants per replicate ($n = 30–90$). Mean +/− standard error is shown and letters indicate significant ($p < 0.05$) differences as determined by a one-way ANOVA follow by a post-hoc Tukey HSD test. **C** Light microscopy of *P. aeruginosa* PAO1 expressing GFP (green) growing for two days on Arabidopsis roots shows that deletion of *colR* leads to visibly less PAO1 colonization of the root. **D**, **E** Wild-type *P. aeruginosa* LESB58 and a LESB58 Δ*colR* mutant were injected (~5 ×10^7 CFU inoculum) into the subcutaneous thin skeletal muscle on the dorsum of mice. CFU counts (**E**) and abscess lesion size (**E**) were determined three days post infection. Means +/− standard error are shown; each dot represents the results from one animal. Different letters indicate significant differences at $p < 0.05$ determined using a one-way ANOVA followed by a Tukey's HSD test, or using a *t*-test if only one comparison was made. **F** Representative images of abscess formation by wild-type LESB58 and the Δ*colR* mutant.

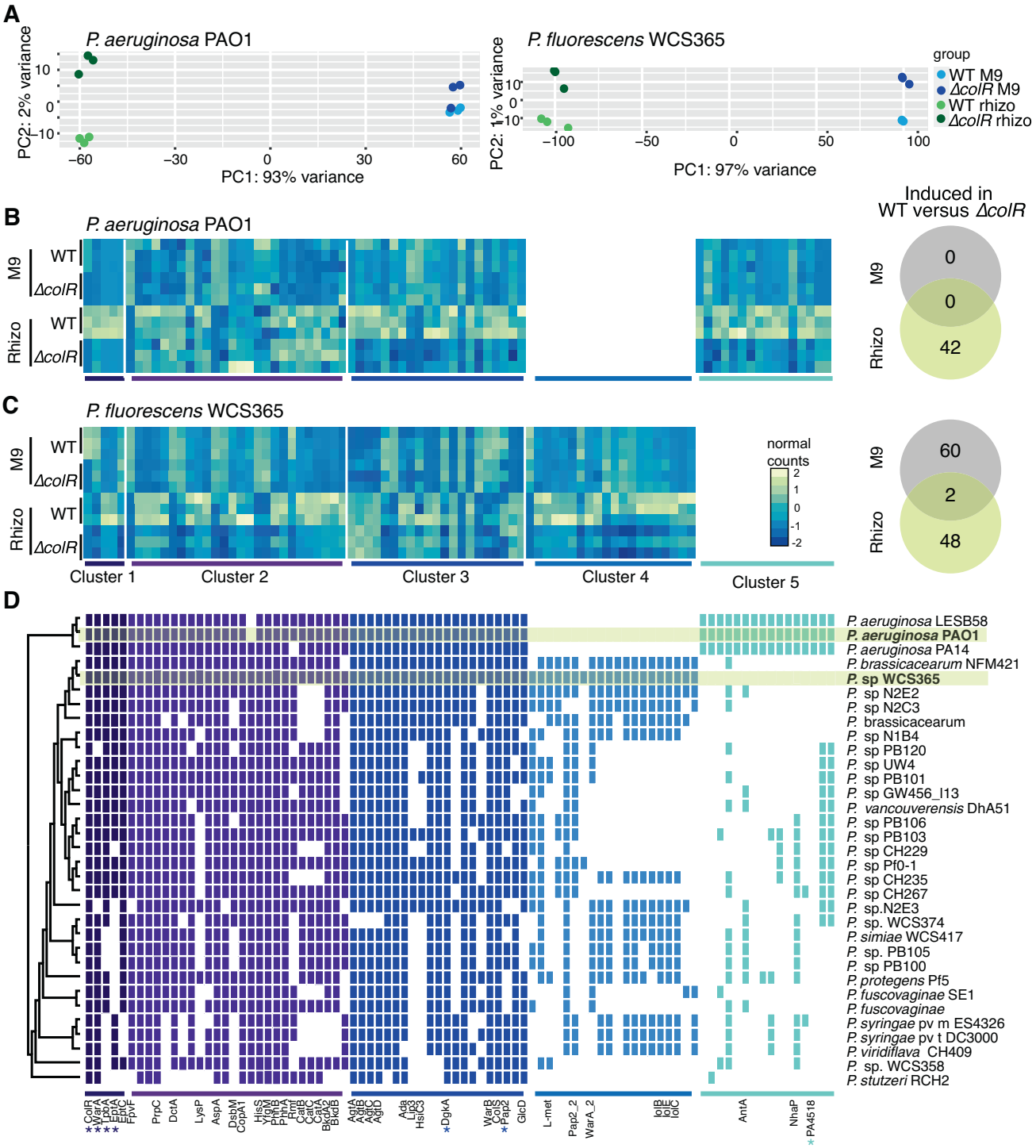

Fig. 2 ColR regulates a limited number of genes in the rhizosphere that are distinct between *Pseudomonas* strains. A PCA plots of transcript count matrices of RNA-Seq analysis comparing gene expression in wild-type *P. aeruginosa* PAO1 and the PAO1 Δ*colR* mutant (left) or *P. fluorescens* WCS365 and the WCS365 Δ*colR* mutant (right). Heat maps and Venn diagrams showing the number of significantly differentially expressed genes (greater than 0.585-fold change, padj <0.01) between wild-type and the *colR* mutant in the rhizosphere or in minimal media in PAO1 (**B**) and WCS365 (**C**). Gene counts higher than row average are shown in yellow, and gene counts lower than row average are shown in blue. **D** A phylogenetic tree showing orthologous genes across *Pseudomonas* strains. Genes fell into 5 distinct clusters: Cluster 1. Genes with ColR-dependent expression in both WCS365 and PAO1; Cluster 2. Genes with ColR-dependent expression in WCS365 with ColR-independent orthologs in PAO1; Cluster 3. Genes with ColR-dependent expression in PAO1 with ColR-independent orthologs in WCS365; Cluster 4. Genes with ColR-dependent in WCS365 without orthologs in PAO1; Cluster 5. ColR-dependent expression in PAO1 without orthologs in WCS365. Genes tested for rhizosphere colonization (Fig. 3) are marked with an asterisk.

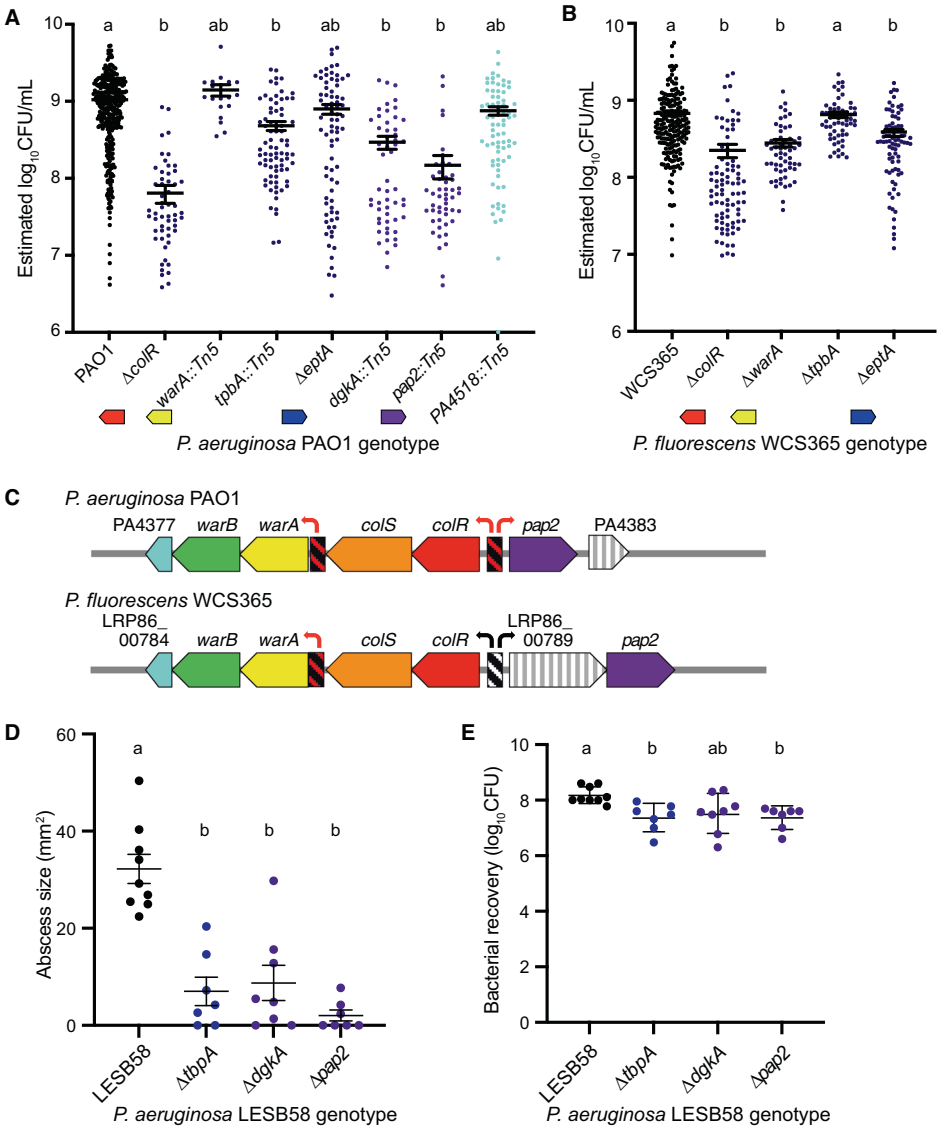

Fig. 3 Strain-specific ColR-dependent genes are required for both rhizosphere colonization and virulence in a mouse abscess model. **A** Wild-type *P. aeruginosa* PAO1 or PAO1 mutants [*ΔcolR*, *warA*::Tn5 (PA4379), *tpbA*::Tn5 (PA3885); *ΔeptA* (PA1927), *dgkA*::Tn5 (PA3603), *pap2*::Tn5 (PA4382), or PA4518::Tn5] and **B** Wild-type *P. fluorescens* WCS365 or WCS365 mutants [*ΔcolR*, *ΔwarA* (LRP86_00786), *ΔtpbA* (LRP86_02280), or *ΔeptA* (LRP86_03626)] containing GFP-expressing plasmids were inoculated into the Arabidopsis rhizosphere of hydroponically grown seedlings. Each data point represents the estimated cfu/mL for a single plant. Three independent experiments, each with a minimum of 10 plants, were performed ($n > 30$). Letters indicate significant differences at $p < 0.05$ using an ANOVA and Tukey's HSD. Colors correspond to the gene clusters shown in Fig. 2D. **C** Genomic arrangement of orthologous genes in *P. fluorescens* WCS365 and *P. aeruginosa* PAO1. Orthologous genes are shown in the same color. Red arrows and striped, red boxes indicate putative ColR-binding sites and ColR-dependent transcription. Black arrows indicate ColR- independent transcription. D and E Wild-type *P. aeruginosa* LESB58 or LESB58 mutants [*ΔdgkA* (PALES_14321), *ΔtpbA* (PALES_10921), and *Δpap2* (PALES_47610)] were injected into the subcutaneous thin skeletal muscle on the dorsum of mice. Abscess lesion size (**D**) and CFU counts (**E**) were determined three days post-infection. Error bars represent standard deviation. Letters indicate significant differences at $p < 0.05$ as determined by a Mann-Whitney U test; each dot represents the results from one animal.

or generated clean deletions (PAO1 *ΔeptA* and WCS365 *ΔeptA, ΔtpbA*, and Δ*warA*). We found that the *tpbA*::Tn5 mutant had reduced rhizosphere colonization in PAO1 but deletion of the WCS365 *tpbA* ortholog had similar rhizosphere colonization as wild-type WCS365 (Fig. 3A, B). While the WCS365 Δ*eptA* and Δ*warA* mutants had reduced rhizosphere colonization, the PAO1 *ΔeptA* and *warA*::Tn5 mutants did not (Fig. 3A, B). None of the mutants had growth defects in vitro indicating that they are specifically required for host association (Supplementary Fig. S5). Collectively these data indicate that while ColR is a conserved determinant of rhizosphere colonization, ColR-dependent genes required for rhizosphere colonization have diverged between *P. fluorescens* WCS365 and *P. aeruginosa* PAO1.

We investigated if *colR*-dependent genes required for *P. aeruginosa* PAO1 rhizosphere colonization were also required for virulence in the murine subcutaneous abscess model. To find additional ColR-dependent genes required for rhizosphere colonization and abscess formation in *P. aeruginosa*, we tested rhizosphere colonization by 25 transposon insertion mutants in PAO1 genes with ColR-dependent expression (Supplementary Dataset S2; Methods). From this screen we identified two additional mutants, *dgkA* (encoding a diacyl glycerol kinase) and *pap2* (with similarity to type 2 family phosphatidic acid phosphatases), that had significantly decreased colonization in the rhizosphere (Fig. 3A) but did not have growth defects in vitro (Supplementary Fig. S5).We found that deletion of the *P.*

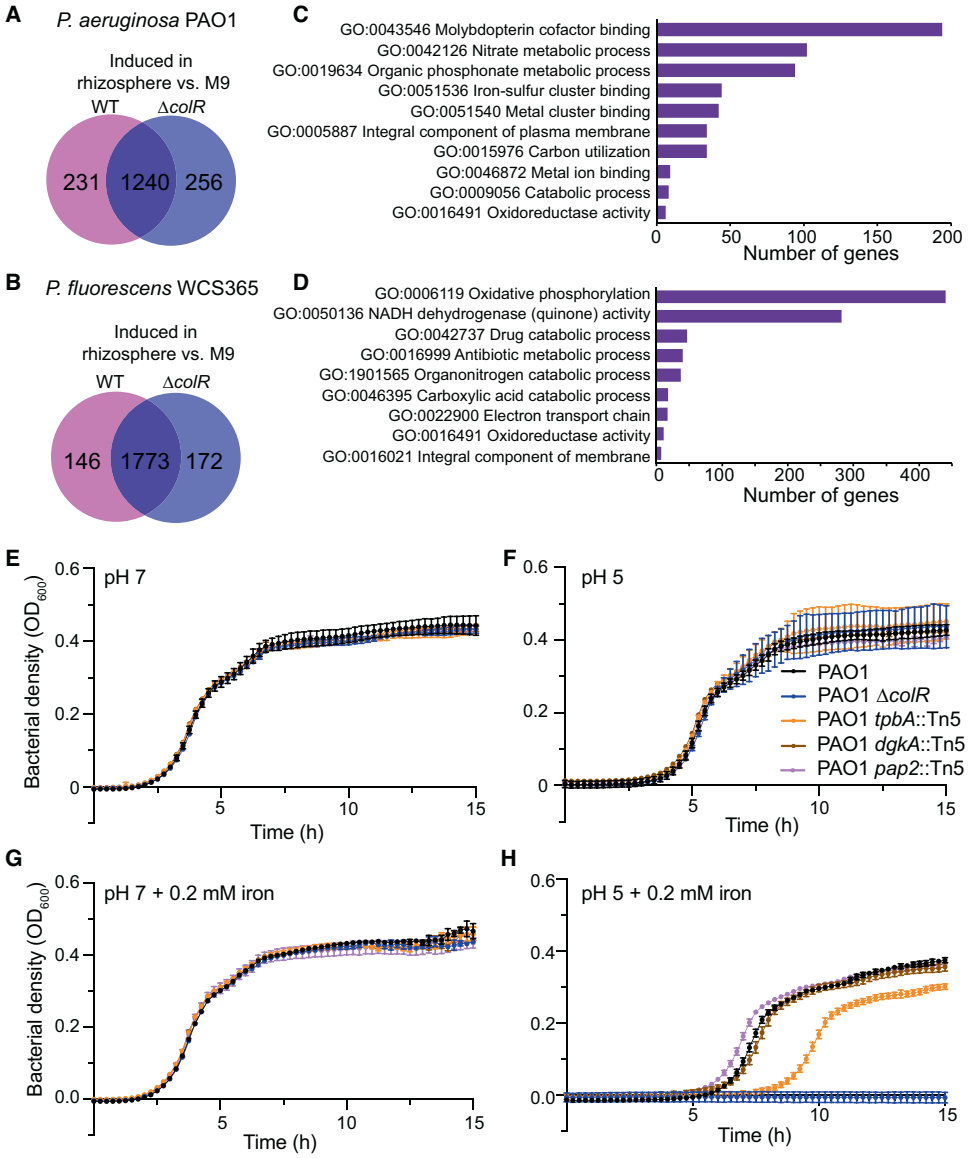

Fig. 4 The *P. aeruginosa* PAO1 *ΔcolR* mutant is sensitive to low pH and high iron. Venn diagrams of the number of total genes in PAO1 (**A**) and WCS365 (**B**) that are significantly upregulated in wild-type or a *colR* mutant in the rhizosphere vs. M9 minimal media (greater than 0.585-fold change, padj < 0.01). **C, D** GO categories of significantly differentially expressed genes between the rhizosphere and M9 minimal media in wild-type PAO1 (**C**) and wild-type WCS365 (**D**). Significantly enriched GO terms were identified using GOfuncR. **E**–**H** Growth curves of wild-type PAO1 and PAO1 mutants (*ΔcolR*, *tpbA*::Tn5, *dgkA*::Tn5 or *pap2*::Tn5) in LB at pH 5 or 7 with and without the addition of 0.2 mM $FeSO_4$. Growth curves were performed at least three times with similar results. Single experimental replicates are shown with averages and standard deviations of 6 technical replicates per time point.

aeruginosa LESB58 orthologs of the genes with rhizosphere colonization defects in PAO1 (*dgkA*, *pap2*, and *tpbA*), resulted in decreased virulence in the abscess model (Fig. 3D). In addition, deletion of *tpbA* and *pap2* led to a small but significant decrease in bacterial recovery (Fig. 3E), indicating that virulence of these mutants was impaired. Collectively these data indicate that an overlapping set of ColR-dependent genes is required for *P. aeruginosa* to associate with diverse hosts.

Transcriptional profiling suggests *Pseudomonas* strains experience diverse stresses in the rhizosphere

We found that the largest differences in gene expression were due to differences in bacterial growth condition (minimal media vs. rhizosphere, Fig. 2A) rather than differences in genotype. To identify rhizosphere stressors encountered by *Pseudomonas* spp. that might reveal why ColR is necessary for rhizosphere colonization, we queried categories of genes that are induced in the rhizosphere in wild-type bacteria (Fig. 4A, B) using gene ontology (GO) analysis. We found that in both *P. fluorescens* WCS365 and in *P. aeruginosa* PAO1, the global changes in gene regulation were largely similar between wild-type bacteria and a *colR* mutant in the rhizosphere when compared to minimal media (Fig. 4A, B; Supplementary Dataset S3). In both *Pseudomonas* species, we observed enrichment of genes involved in detoxification and survival in a harsh environment. These include metabolism of antibiotics and organic compounds, and genes involved in detoxifying the environment and responding to a new environment (Fig. 4C, D; Supplementary Dataset S3). The upregulation of genes involved in stress tolerance in both WCS365 and in PAO1 suggested that to grow in the rhizosphere, bacteria must protect themselves against harmful rhizosphere components.

Because we found upregulation of GO terms and processes related to protection against environmental stressors and membrane modifications, we tested if known rhizosphere stressors, including the presence of cationic peptides [34], could explain the growth inhibition of a *colR* mutant. We found that the minimal inhibitory concentration (MIC) of the cationic peptide polymyxin B was similar between wild-type PAO1 and the *colR* mutant (Supplementary Fig. S6). We also tested biofilm formation in a *colR* mutant, as biofilm formation was previously shown to be increased in a *colR* mutant, and is important for virulence [35] and rhizosphere colonization [36]. However, the *colR* mutant formed slightly lower levels of biofilm as wild-type bacteria in PAO1 and did not form lower levels of biofilm in LESB58 (Supplementary Fig. S7). This could be due to the use of different *P. aeruginosa* isolates (PA14 vs. PAO1 and LESB58), or the different conditions under which biofilm formation was tested (endotracheal tube vs. 96-well plate). These data indicate that the defect in rhizosphere growth of a *colR* mutant is unlikely to be due to the presence of cationic peptides or a change in biofilm formation.

ColR is necessary for growth at low pH and resistance to transition metals

We found that genes induced in the rhizosphere are enriched for GO terms including metal ion binding and electron transfer activity (Fig. 4), suggesting that bacteria experience metal stress in the rhizosphere. Previous studies showed that a *P. putida colR* mutant is sensitive to high levels of zinc and iron, and that this metal sensitivity is dependent on the loss of multiple ColR-dependent membrane modifying genes [21, 32]. We therefore confirmed that a *P. aeruginosa* PAO1 Δ*colR* mutant is similarly sensitive to metals. We determined the minimum inhibitory concentration (MIC) of PAO1 and the Δ*colR* mutant. We found that the *P. aeruginosa* PAO1 Δ*colR* mutant has lower tolerance to $ZnSO_4$ (WT MIC = 10 mM; Δ*colR* MIC = 5 mM) and $FeSO_4$ (WT MIC = 5 mM; Δ*colR* MIC = 2.5 mM) and similar tolerance to $MnSO_4$ (WT MIC = 10 mM; Δ*colR* MIC = 10 mM) and $CuSO_4$ (WT MIC = 10 mM; Δ*colR* MIC = 10 mM) than wild-type PAO1. Using metal concentrations below the MIC of *P. aeruginosa* PAO1 Δ*colR*, we performed growth curves with PAO1 and the Δ*colR* mutant and found that a PAO1 *colR* mutant has reduced growth relative to wild-type PAO1 in the presence of zinc, iron, and manganese (Supplementary Fig. S8).

The concentrations of metals in 1/2 X MS media (15 μM Zn, 0.1 mM Fe, and 0.05 mM Mn), the media in which the plants are grown, is below the levels that inhibit the Δ*colR* mutant (3 mM Zn, 1 mM Fe, and 3 mM Mn); however, metal solubility increases at low pH, and the rhizosphere is acidic [22] so we hypothesized that a synergistic effect of pH and metal stress might be inhibitory [37]. We measured bacterial growth at pH 5 in the presence of 0.2 mM $FeSO_4$ and found that *colR* is required for tolerance to this combination of metal and pH stress (Fig. 4H). We tested if *P. aeruginosa tpbA*::Tn5, *dgkA*::Tn5 and *pap2*::Tn5 mutants also had increased sensitivity to $FeSO_4$ at low pH and found that *tpbA*::Tn5 but not the other mutants partially phenocopied the growth defect of the Δ*colR* mutant (Fig. 4H). We also measured bacterial growth at pH 5 in ½ X MS plant growth media with succinate as the sole carbon source and varying concentrations of $FeSO_4$.We found that both wild-type PAO1 and a Δ*colR* mutant have shorter doubling times in 1/2X MS media containing an additional 0.25 mM $FeSO_4$ at pH 5 and that a Δ*colR* mutant has a consistent delay in growth relative to wild-type bacteria under this condition (Supplementary Fig. S9F, H). These data indicate that there is a complex interaction between media composition, pH, and metal concentration. These data also indicate that either the loss of multiple ColR-dependent genes is required for sensitivity to the synergetic effects of 0.2 mM $FeSO_4$ and low pH, or that the loss of a ColR-dependent gene which we did not test is required for this sensitivity.

A mechanism of defence present in both plants and animals is the generation of reactive oxygen species (ROS) using iron through Fenton chemistry [38, 39] suggesting a potential shared environment across hosts that might require ColR. We tested whether the ColR mutant has increased sensitivity to ROS using H_2O_2 and found that while a *katA*::Tn5 mutant unable to make catalase cannot grow in 20 mM H_2O_2, growth of the *P. aeruginosa* PAO1 Δ*colR* mutant is indistinguishable from wild-type bacteria (Supplementary Fig. S10A). Additionally, survival of a Δ*colR* mutant after 30 min of treatment with 1 or 20 mM H_2O_2 was similar to wild-type *P. aeruginosa* (Supplementary Fig. S10B). As zinc and manganese do not participate in Fenton chemistry, and the PAO1 Δ*colR* mutants had dysregulation of genes involved in membrane modification (*eptA, pap2,* and *dgkA*) and ion transporters (*mntP, fecA, and copA1*), this suggests *colR* mutants might have increased uptake and decreased efflux of metals leading to toxicity.

DISCUSSION

Bacteria in the genus *Pseudomonas* can form symbiotic associations with a diverse range of hosts with outcomes ranging from mutualism to pathogenesis. We showed that ColR is a conserved symbiosis factor, required for host-association not only in diverse *Pseudomonas* species, but also in diverse hosts. We found that the ColR regulon, and the ColR-dependent genes required for host association in distinct *Pseudomonas* spp., are largely divergent. Only four orthologous genes, *eptA, eptC, warA*, and *tpbA*, are ColR-dependent in both *P. fluorescens* WCS365 and *P. aeruginosa* PAO1. Of the remaining ColR-dependent genes, some were conserved between *P. fluorescens* WCS365 and *P. aeruginosa* PAO1 with divergent regulation, while others were not conserved between strains. This indicates that the divergence of the ColR regulon between strains may be due to both gain and loss of accessory (non-conserved) genome components, or promoter evolution, for example through the loss or gain of a ColR binding site in the promoter. Due to the conservation of ColR DNA binding residues, and ability to complement a WCS365 Δ*colR* mutant with PAO1 *colR*, it seems unlikely that changes to the ColR protein itself underly differences in the ColR regulon between the two strains. We found that none of the four conserved ColR-dependent genes are required for rhizosphere colonization in both species. This indicates that ColR/S may play a conserved role in sensing the host and responding through changes in transcription; however, the specific transcriptional changes depend on the bacterial genetic background.

We performed transcriptional profiling and GO analysis of genes upregulated in the rhizosphere in comparison to M9 minimal media, to identify genes that are likely involved in adaptation to growth in the rhizosphere environment (Fig. 4). By identifying stressors that bacteria experienced in the rhizosphere, we found that a Δ*colR* mutant is sensitive to a combination of low pH and high iron levels, suggesting a mechanism by which the rhizosphere or an abscess might inhibit growth or virulence, respectively, of a *colR* mutant. Metals are more soluble at low pH [37] and have diverse mechanisms of toxicity including mismetallation, inhibition of electron transport, and antagonizing uptake of other essential metals [40]. We found that the PAO1 Δ*colR* mutants had dysregulation of genes involved in membrane modification (*eptA, pap2,* and *dgkA*) and ion transport (*mntP, fecA, and copA1*; Fig. 2D and Supplementary Dataset S2), suggesting that *colR* mutants might have increased membrane permeability resulting in higher cytosolic metal levels. Coupled with decreased expression of efflux transporters this may lead to metal toxicity in a *colR* mutant.

As *P. aeruginosa pap2* and *dgkA* mutants were impaired in rhizosphere colonization and virulence, but were not sensitive to low pH and high metals, this indicates that there must be an additional stress or process that ColR is required to adapt to in

both the plant rhizosphere and in the murine abscess. This could be an inhibitory antimicrobial peptide, lack of an essential nutrient, or a heightened immune response in the plant rhizosphere or mouse tissue. DgkA and Pap2 convert diacylglycerol (DAG) to phosphatidic acid (PA), and PA to DAG, respectively [41, 42]. Both PA and DAG are key intermediates and precursors to the synthesis of many major membrane phospholipids [42]. Additionally, DAG is generated as a result of phosphoethanolamine (pEtN) addition to Lipid A [43]. We identified three pEtN transferase enzymes that were ColR-dependent in PAO1 [*eptA* (PA1972), *eptA2* (PA3310) and *eptC* (PA4517)] and two in WCS365 [*eptA* (LRP86_03626) and *eptC* (LRP86_05619)]. This suggests that *dgkA* may be required for turnover of the resulting DAG and that loss of *colR* may result in decreased membrane structural integrity through disruption of phospholipid synthesis and turnover.

This study has found that *colR* is required for rhizosphere colonization in *P. fluorescens* and in *P. aeruginosa* and abscess formation in *P. aeruginosa* but not through the regulation of conserved genes. This suggests that ColS may be a conserved host-sensing protein that activates ColR to adapt to a host environment; however, the ColR regulon may have diverged according to bacterial lifestyle. Divergence of two-component system regulons has been previously shown to occur in members of the family *Enterobacteriaceae*, in which the PhoP response regulator targets were largely divergent among species [44]. Similarly, virulence through type III secretion systems relies on a conserved secretion apparatus coupled with host-specific diversification of effectors. This suggests that conservation of two-component systems, coupled with lifestyle dependent diversification of the regulon, may play a role in host association and lifestyle transitions.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions

Bacteria strains and plasmids are listed in Supplementary Dataset S1. All transposon mutants were obtained from the PAO1 two-allele library [33]. Routine bacterial culturing was performed using LB broth or agar at 37 °C (for *P. aeruginosa* PAO1 and LESB58 as well as *E. coli*) or at 28 °C (for *P. fluorescens* WCS365). When appropriate, LB was supplemented with sucrose, 25 µg/ml kanamycin (Km), 15 µg/ml gentamicin (Gm), 200 µg/ml carbenicillin (Cb), 10 µg/ml nalidixic acid, and/or 25 µg/ml irgasan.

Strain construction

E. coli DH5α was used for construction and maintenance of plasmids, and *E. coli* SM10 λpir was used for biparental conjugation with *P. aeruginosa* and *P. fluorescens*. Deletion mutants were generated using the two-step allelic exchange method [17, 45–47]. Deletion constructs for PAO1 Δ*colR* (PA4318), Δ*eptA* (PA1972), LESB58 Δ*colR* (PALES_47611), Δ*tpbA* (PALES_14321), Δ*dgkA* (PALES_47610), Δ*pap2* (PALES_10931), WCS365 Δ*colR* (LRP86_00788), Δ*eptA* (LRP86_03626), Δ*warA* (LRP86_00786), and Δ*tpbA* (LRP86_02280) were generated using suitable primers (Supplementary Dataset S1). As the flanking regions have 100% sequence identity, the same deletion construct for Δ*colR* was used to delete *colR* from both PAO1 and LESB58.

Briefly, approximately 700–800 bp of sequence flanking the target genes were amplified using PCR. The flanking regions were joined using overlap extension PCR [48]. The PCR products were digested using the appropriate restriction enzymes (Supplementary Dataset S1), and ligated into the multiple cloning site of pEXG2 suicide vector containing *sacB* [49]. After confirming the insert by Sanger sequencing, the vector was transformed into *E. coli* SM10 λpir and transformants containing the plasmid were selected for on LB agar plates supplemented with 15 µg/ml Gm. The plasmid was then transferred to *Pseudomonas* via conjugation into PAO1, LESB58, or WCS365, and integrated into the genome through homologous recombination. Merodiploids were selected using Gm (50 µg/ml for PAO1, and 500 µg/ml for LESB58) and irgasan (25 µg/ml) for *P. aeruginosa*, or Gm (15 µg/ml) and nalidixic acid (10 µg/ml) for *P. fluorescens*. Counter-selection on LB plates with 10% sucrose was performed to select for loss of the plasmid. PCR and Sanger sequencing was used to screen for and validate deletion mutants.

The *colR* complementation plasmids were created using the pBBR1MCS-5 plasmid as a backbone for PAO1 and pBBR1MCS-2 for WCS365. PAO1 *colR*, or WCS365 *colR*, and approximately 200 bp upstream of each gene, respectively, were amplified using corresponding primers (Supplementary Dataset S1). Amplicons and plasmids were digested with the appropriate enzymes and then ligated together, and the resulting insert was confirmed by Sanger sequencing. The *colR* complementation strains were generated by electroporating pBBR1MCS-5-PAO1colR (PcolR) into the PAO1 Δ*colR* mutant, and pBBR1MCS-2-WCS365*colR* (W*colR*) into the WCS365 Δ*colR* mutant. Gm (PAO1) or Km (WCS365) were used for selection and maintenance of the plasmid. Wild-type and Δ*colR* with empty vector (EV) were generated by electroporation with pBBR1MCS-5 for PAO1 or pBBR1MCS-2 for WCS365. GFP-expressing *P. aeruginosa* and *P. fluorescens* were generated by electroporating strains with pSMC21 (Ptac-GFP) [50, 51]. Electrocompetent cells were prepared by pelleting, washing and resuspending cultures in 300 mM sucrose. Transformants were selected on and maintained using Cb (300 µg/ml) for *P. aeruginosa*, and Km (25 µg/ml) for *P. fluorescens*.

Plant growth conditions

Axenic plants were generated by surface sterilizing Arabidopsis (wild-type accession Col-0) seeds in 70% ethanol for approximately 2 min, 10% bleach for approximately 2 min, and washed three times with sterile water (H_2O). Seeds were resuspended in H_2O or 0.1% phytoagar and stored at 4 °C in the dark for at least 48 h before sowing on Murashige and Skoog (MS) liquid media [52], as described below. Plants were grown at 16 h light/8 h dark under 100 µM cool white fluorescent lights at 22 °C.

Rhizosphere colonization in 48-well plates

Plants were grown hydroponically in flat-bottom 48-well plates as described [17, 30]. Briefly, one autoclave-sterilized Teflon mesh disk was placed in each well of a 48-well plate containing 250–300 µl MS plant growth media supplemented with 2% sucrose at pH 5.8. Sterilized Arabidopsis seeds were placed individually in the center of each disk. The plant media was changed to 270 µl of ½X MS media at pH 5.8 without sucrose after 10 days. At 12 days, overnight cultures of GFP-expressing bacteria were diluted to an OD_{600} of 0.0002 in 10 mM $MgSO_4$, and 30 µl was inoculated into each well. GFP fluorescence (485 nm/535 nm excitation/emission) was read from the bottom of the wells at 5 days post-inoculation, using a SpectraMax i3x fluorescent plate reader (Molecular Devices). Bacterial growth in a minimum of 10 plant root-containing wells was measured for each strain per experiment.

Bacterial abundance in the rhizosphere was estimated from the GFP fluorescence readings of the entire well containing bacteria free-living in the well and those attached to the root and reported in CFU per ml by first converting GFP fluorescence readings to OD_{600} using standard curves specific to each strain or mutant, and then converted to CFU per ml using known values of number of bacteria per OD_{600} for both *P. fluorescens* WCS365 and *P. aeruginosa* PAO1. Standard curves of GFP fluorescence vs. OD_{600} were constructed for *P. aeruginosa* PAO1 and *P. fluorescens* WCS365. Separate standard curves were constructed for PAO1 and WCS365 strains that harbor two plasmids (pSMC21 and pBBR1MCS-5 or pBBR1MCS-2), because of possible differences in plasmid copy number due to the presence an additional expression vector. These plasmids contain a stability fragment that has been shown to confer a high degree of plasmid stability in the absence of selection [50].

Screening of the Manoil lab PAO1 two-allele library transposon insertion mutants (Supplementary Dataset S2) for rhizosphere colonization defects were performed similarly, but OD_{600} of the liquid media from each well was measured instead of GFP fluorescence. Non-GFP-expressing bacteria were inoculated into wells containing 12-day-old plants to a final OD_{600} of 0.0002. At 5 days post inoculation, 80 µl of media were transferred from each plant well into a 96-well plate. PAO1 and PAO1 Δ*colR* were included in each experiment as controls. OD_{600} readings were taken from the 96-well plate using SpectraMax i3x. A minimum of 8 wells were measured per strain. The ratio of mutant to wild-type colonization was estimated by dividing mutant OD_{600} over PAO1 OD_{600} after subtraction of the blank reading. Mutants with a OD_{600} ratio of below 0.7 relative to PAO1 were considered candidates for rhizosphere colonization defects and were retested using the GFP method above.

Study approval and animals

Animal experiments were performed in accordance with the Canadian Council on Animal Care (CCAC) guidelines following approval by the

University of British Columbia Animal Care Committee (A14-0253). Mice used in this study were outbred CD-1 mice (female, 7–8 weeks). All animals were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Mice weighed 25 ± 5 g at the experimental endpoint. Animals were group housed in cohorts of 4–5 littermates exposed to the same pathogen. Littermates were randomly assigned to experimental groups and standard animal husbandry protocols were employed.

Murine subcutaneous abscess infection model

The role of ColR in virulence during high density infection was examined using *P. aeruginosa* LESB58 [46] in a murine subcutaneous abscess model [29]. Briefly, the LESB58 wild-type and mutant strains (Δ*colR*, Δ*dgkA*, Δ*pap2*, Δ*tpbA*) were sub-cultured at 37 °C with shaking (250 rpm) to an $OD_{600} = 1.0$ in LB. Cells were washed twice with sterile phosphate buffered saline (PBS) and resuspended to a final $OD_{600} = 1.0$. Bacteria were injected (50 µl) subcutaneously into the right or left dorsum of mice for an inoculum density of ~5.0 $\times 10^7$ CFU. Abscesses were formed for 72 h with daily clinical grading. At the experimental endpoint, mice were euthanized by CO_2 and cervical dislocation. Visible dermonecrosis was measured using a caliper, and abscesses were harvested in PBS. Then abscess tissues were homogenized using a Mini-Beadbeater (BioSpec Products, Bartlesville, OK) for bacterial enumeration on LB following serial dilution. Two or three independent experiments containing 2–4 biological replicates each were performed ($n = 6$–12).

Bacterial RNA extraction and RNA-Seq

RNA from rhizosphere-growing and minimal media-growing *P. aeruginosa* PAO1 and *P. fluorescens* WCS365 was extracted for RNA sequencing and analysis. M9 minimal media supplemented with 24 mM L-glutamine (L-gln) (*P. aeruginosa* PAO1), or supplemented with 30 mM sodium succinate (*P. fluorescens* WCS365) was used as the minimal media control. Plants were grown hydroponically as described in the 48-well plate rhizosphere colonization assay. Wells containing 12-day-old plants were inoculated with the wild-type and *colR* deletion mutants of *P. aeruginosa* PAO1 and *P. fluorescens* WCS365. To obtain sufficient RNA for sequencing, 6 wells containing plants or minimal media were inoculated with a final OD_{600} of 0.2 for PAO1 and 0.1 for WCS365. Both plant and minimal media plates were incubated in the plant growth conditions described above for 6 h. After 6 h, 40 µl of media from each of 6 plant wells or media from 3 minimal media wells were pooled and stabilized in RNAprotect Bacteria Reagent (QIAGEN) before performing RNA extraction using RNeasy Mini Kit (QIAGEN). When necessary, RNA was concentrated using ethanol precipitation.

RNA from three biological replicates was used with RNA integrity numbers (RIN) > 9.9. cDNA library preparation and RNA sequencing, using paired-end 150 bp reads, were performed by GENEWIZ using HiSeq (Illumina). The sequencing yielded an average of 16,897,599 (standard deviation = 429,191) reads for each sample for all PAO1 and PAO1 Δ*colR* samples, and an average of 16,055,232 (standard deviation = 760,587) reads for each sample for all WCS365 and WCS365 Δ*colR* samples. The quality of the reads was assessed using FASTQC v.0.11.8 [53]. Salmon v.1.1.0 [54] was used to align reads to the PAO1 transcriptome and to obtain the count files for each sample. Reads were mapped to the PAO1 transcriptome with an average mapping rate of 70.8% (standard deviation = 1.3%) for each sample for PAO1 and were mapped to the WCS365 transcriptome with an average mapping rate of 75.5% (standard deviation = 3.5%) for each sample for WCS365. DESeq2 v1.26.0 was used for differential expression analysis in R [55]. Log_2 fold change values were corrected using the *apeglm* package in R [56]. Genes with a log_2 fold change of ≥1.5 (±0.585 fold change) and adjusted p value ≤ 0.01 were considered significantly differentially expressed. Normalized reads for each gene were generated using the DESeq2 package in R. The dplyr package was used for data manipulation (sorting and merging lists). The heatmap function in R was used to generate a heatmap using reads normalized for length of each gene.

Comparative genomics

Ortholog groups between the ColR-dependent genes in WCS365 and PAO1 were identified using the PyParanoid pipeline described previously [1]. Briefly, the PyParanoid pipeline was previously used to create a database using 3886 *Pseudomonas* genomes, identifying 24,066 homologous protein families ("gene groups") that covered 94.2% of the generated *Pseudomonas* pangenome. Groups were identified based on the PAO1 or WCS365 amino acid sequences and mapped onto a phylogenetic tree (Fig. 2). Phylogenetic trees were generated using the PyParanoid pipeline as described previously [1, 57].

Functional analysis of RNA-seq results

Gene ontology (GO) terms assigned to WCS365 were assigned using Blast2GO [58]. Lists of genes significantly differentially expressed in each condition or genotype were created using DEseq2, as described above. Lists of genes with assigned GO terms were inputted into R. GOfuncR [59] was used to identify GO terms significantly enriched in the conditions inputted (wild-type bacteria or Δ*colR* in the rhizosphere or minimal media). The go_enrich command was used to identify significantly enriched GO terms and GO terms with a FWER (family-wise error rate) ≤0.1 were refined to include only those terms with a refined p value ≤ 0.05. Code is available on the Haney Lab GitHub page: https://github.com/haneylab/ColR_paper_RNAseq_analysis.

In vitro metal, pH and H_2O_2 sensitivity assays

For all growth curves, bacterial cultures were pipetted into clear, flat-bottom 96-well plates to a final OD_{600} of 0.02 and a final volume of 100 or 200 µl. Growth was monitored using a shaking plate reader (Molecular Devices VersaMax or SpectraMax microplate reader), at a fixed temperature of 37 °C for PAO1 or 28–30 °C for WCS365. OD_{600} readings were taken once every 15 min for 24 h. At least four technical replicates were used for each strain and condition. The growth curves for each combination of strain and condition were repeated three times.

Growth in metals or H_2O_2 were assessed by addition of the appropriate concentration of metals or H_2O_2 to LB ($FeSO_4$, $ZnSO_4$, $MnSO_4$, and $CuSO_4$) adjusted to pH 5 or 7 with HCl or KOH. Metal concentrations used were below the MIC for both PAO1 and PAO1 Δ*colR*. To determine the MIC of the transition metals tested, LB media supplemented with 2X the highest concentration of metals ($ZnSO_4$, $MnSO_4$, $FeSO_4$, and $CuSO_4$) to be used for the MIC assay were prepared. The solutions of LB + metal were then serially diluted by 2X down the rows of 96-well plates. The final volume in each well was 50 µl in LB or 200 µL in 1/2X MS. Overnight bacterial cultures in LB were resuspended in fresh LB and diluted to an OD_{600} of 0.04 or to an OD_{600} 0.1 in 1/2X MS. 50 µl (LB) or 200 µL (1/2X MS) of diluted bacterial cultures were added to each well. The 96-well plates were placed in an incubator at 37 °C for 24 h (LB) or 12 h (1/2X MS) before assessment of MIC. The MIC values were determined by eye (LB) or by dilution plating (1/2X MS) and scored as the lowest concentration of metals in which there was at least a 50% reduction in growth. Percent survival in H_2O_2 was performed by diluting overnight cultures 1:100 to an OD_{600} of 0.04–0.08 and adding H_2O_2 to a final concentration of 1 or 20 mM. After a 30 min incubation, bacteria were plated on LB plates and % survival was calculated relative to time 0 for each genotype and replicate.

Growth curves in ½X MS or M9 were performed as described above. Succinate at 20 mM was used as the carbon source in M9 minimal media and 1/2X MS media. For growth curves at varying pH, media was adjusted to pH 5, 6, or 7 using KOH. Overnight bacterial cultures in LB were washed and resuspended in 10 mM $MgSO_4$ and diluted to an OD_{600} of 0.2. 90 µl of sterile media were aliquoted into each well in a 96-well plate before inoculation with 10 µl of the diluted bacterial suspension.

Polymyxin B MIC assays

The MIC assay for PAO1 was performed according to a modified MIC method for antimicrobial peptides (http://cmdr.ubc.ca/bobh/method/modified-mic-method-for-cationic-antimicrobial-peptides/). Briefly, individual bacterial colonies grown on MH agar media were used to start overnight cultures in MH broth. Serial dilutions of polymyxin B were performed by making 20X solutions of the antibiotic in 0.01% acetic acid, 0.2% BSA and serially diluting the antibiotic two- fold into sterile 96-well polypropylene microtitre plates. Cultures were then diluted to 5 $\times 10^6$ cfu/mL ($OD_{600} = 0.0073$) and 100 µL of this diluted culture was added to each well. The MIC values were determined by eye and scored as the lowest concentration of polymyxin B in which there was at least a 50% reduction in growth.

Biofilm assay

P. aeruginosa PAO1 strains were tested in 24 h biofilm assays quantifying total biofilm biomass [60]. Briefly, bacteria from overnight cultures were diluted to an OD_{600} of 0.05 in Synthetic Cystic Fibrosis Media [61] without ammonium chloride (PAO1 strains) or LB (LESB58 strains) and 100 µl were

inoculated into 96 well plates. After static growth for 24 h at 37 °C, the planktonic bacteria were rinsed off with deionized water. Biofilm biomass was stained with 105 µl 0.1% crystal violet and destained with 110 µl 70% ethanol. Lastly, absorbance was measured at 595 nm. Absorbance of each sample was blanked by subtracting the average absorbance of the sterility controls, and then normalized to the average absorbance of the wild-type and expressed as %wild-type biofilm. Statistical significance was determined using an unpaired t-test if comparing two values, or a one-way ANOVA followed by a post-hoc Tukey's HSD test of multiple comparisons to correct for multiple comparisons if more than one value was being compared. p values <0.05 were considered significant.

DATA AVAILABILITY

The *P. fluorescens* WCS365 genome assembly and annotation have been deposited in the National Centre for Biotechnology Information BioProject database under accession CP089973. The RNA-Seq raw sequencing has been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database under accession GSE190448.

CODE AVAILABILITY

The code used for RNA-Seq analysis is available from the Haney laboratory GitHub repository https://github.com/haneylab/ColR_paper_RNAseq_analysis.

REFERENCES

1. Melnyk RA, Hossain SS, Haney CH. Convergent gain and loss of genomic islands drive lifestyle changes in plant-associated *Pseudomonas*. ISME J. 2019;13:1575–88.
2. Savory EA, Fuller SL, Weisberg AJ, Thomas WJ, Gordon MI, Stevens DM, et al. Evolutionary transitions between beneficial and phytopathogenic *Rhodococcus* challenge disease management. eLife. 2017;6:e30925.
3. Sällström B, Andersson SGE. Genome reduction in the α-Proteobacteria. Curr Opin Microbiol. 2005;8:579–85.
4. DeFalco TA, Zipfel C. Molecular mechanisms of early plant pattern-triggered immune signaling. Mol Cell. 2021;81:3449–67.
5. Rahme LG, Ausubel FM, Cao H, Drenkard E, Goumnerov BC, Lau GW, et al. Plants and animals share functionally common bacterial virulence factors. Proc Natl Acad Sci USA. 2000;97:8815–21.
6. Rahme LG, Tan MW, Le L, Wong SM, Tompkins RG, Calderwood SB, et al. Use of model plant hosts to identify *Pseudomonas aeruginosa* virulence factors. Proc Natl Acad Sci USA. 1997;94:13245–50.
7. Clatworthy AE, Lee JS-W, Leibman M, Kostun Z, Davidson AJ, Hung DT. *Pseudomonas aeruginosa* infection of zebrafish involves both host and pathogen determinants. Infect Immun. 2009;77:1293–303.
8. Mulcahy H, Sibley CD, Surette MG, Lewenza S. *Drosophila melanogaster* as an animal model for the study of *Pseudomonas aeruginosa* biofilm infections in vivo. PLOS Pathog. 2011;7:e1002299.
9. Silby MW, Winstanley C, Godfrey SAC, Levy SB, Jackson RW. *Pseudomonas* genomes: diverse and adaptable. FEMS Microbiol Rev. 2011;35:652–80.
10. Vasquez-Rifo A, Veksler-Lublinsky I, Cheng Z, Ausubel FM, Ambros V. The *Pseudomonas aeruginosa* accessory genome elements influence virulence towards *Caenorhabditis elegans*. Genome Biol. 2019;20:270.
11. Wang NR, Wiesmann CL, Melnyk RA, Hossain SS, Chi MH, Martens K, et al. Commensal *Pseudomonas fluorescens* strains protect Arabidopsis from closely related *Pseudomonas* pathogens in a colonization-dependent manner. mBio. 2022;13:e0289221.
12. Guzmán-Verri C, Manterola L, Sola-Landa A, Parra A, Cloeckaert A, Garin J, et al. The two-component system BvrR/BvrS essential for *Brucella abortus* virulence regulates the expression of outer membrane proteins with counterparts in members of the Rhizobiaceae. Proc Natl Acad Sci USA. 2002;99:12375–80.
13. Sola-Landa A, Pizarro-Cerdá J, Grilló M-J, Moreno E, Moriyón I, Blasco J-M, et al. A two-component regulatory system playing a critical role in plant pathogens and endosymbionts is present in *Brucella abortus* and controls cell invasion and virulence. Mol Microbiol. 1998;29:125–38.
14. Bolton GW, Nester EW, Gordon MP. Plant phenolic compounds induce expression of the *Agrobacterium tumefaciens* loci needed for virulence. Science. 1986;232:983–5.
15. Chen EJ, Sabio EA, Long SR. The periplasmic regulator ExoR inhibits ExoS/ChvI two-component signaling in *Sinorhizobium meliloti*. Mol Microbiol. 2008;69:1290.
16. Dekkers LC, Bloemendaal CJ, de Weger LA, Wijffelman CA, Spaink HP, Lugtenberg BJ. A two-component system plays an important role in the root-colonizing ability of *Pseudomonas fluorescens* strain WCS365. Mol Plant Microbe Interact. 1998;11:45–56.
17. Liu Z, Beskrovnaya P, Melnyk RA, Hossain SS, Khorasani S, LR O, et al. A genome-wide screen identifies genes in rhizosphere-associated *Pseudomonas* required to evade plant defenses. mBio. 2020;6:e00433–18.
18. Garvis S, Munder A, Ball G, de Bentzmann S, Wiehlmann L, Ewbank JJ, et al. *Caenorhabditis elegans* semi-automated liquid screen reveals a specialized role for the chemotaxis gene *cheB2* in *Pseudomonas aeruginosa* virulence. PLoS Pathog. 2009;5:e1000540.
19. Jander G, Rahme LG, Ausubel FM. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J Bacteriol. 2000;13:3843–5.
20. Cheng X, Etalo DW, van de Mortel JE, Dekkers E, Nguyen L, Medema MH, et al. Genome-wide analysis of bacterial determinants of plant growth promotion and induced systemic resistance by *Pseudomonas fluorescens*. Environ Microbiol. 2017;19:4638–56.
21. Ainsaar K, Mumm K, Ilves H, Hõrak R. The ColRS signal transduction system responds to the excess of external zinc, iron, manganese, and cadmium. BMC Microbiol. 2014;14:162.
22. Yu K, Liu Y, Tichelaar R, Savant N, Lagendijk E, van Kuijk SJL, et al. Rhizosphere-associated *Pseudomonas* suppress local root immune responses by gluconic acid-mediated lowering of environmental pH. Curr Biol. 2019;29:3913–.e4.
23. Van den Bossche S, De Broe E, Coenye T, Van Braeckel E, Crabbé A. The cystic fibrosis lung microenvironment alters antibiotic activity: causes and effects. Eur Respir Rev. 2021;30:210055.
24. Lugtenberg B, Kamilova F. Plant-growth-promoting rhizobacteria. Annu Rev Microbiol. 2009;63:541–56.
25. Rahme L, Stevens E, Wolfort S, Shao J, Tompkins R, Ausubel F. Common virulence factors for bacterial pathogenicity in plants and animals. Science. 1995;268:1899–902.
26. Attila C, Ueda A, Cirillo SLG, Cirillo JD, Chen W, Wood TK. *Pseudomonas aeruginosa* PAO1 virulence factors and poplar tree response in the rhizosphere. Micro Biotechnol. 2008;1:17–29.
27. Cook J, Douglas GM, Zhang J, Glick BR, Langille MGI, Liu K-H, et al. Transcriptomic profiling of *Brassica napus* responses to *Pseudomonas aeruginosa*. Innate Immun. 2020;27:143–57.
28. Walker TS, Bais HP, Déziel E, Schweizer HP, Rahme LG, Fall R, et al. *Pseudomonas aeruginosa*-plant root interactions. Pathogenicity, biofilm formation, and root exudation. Plant Physiol. 2004;134:320–31.
29. Pletzer D, Mansour SC, Wuerth K, Rahanjam N, Hancock REW. New mouse model for chronic infections by gram-negative bacteria enabling the study of anti-infective efficacy and host-microbe interactions. mBio. 2017;8:e00140–17.
30. Haney CH, Samuel BS, Bush J, Ausubel FM. Associations with rhizosphere bacteria can confer an adaptive advantage to plants. Nat Plants. 2015;1:15051.
31. Cheng Z, Li J-F, Niu Y, Zhang X-C, Woody OZ, Xiong Y, et al. Pathogen-secreted proteases activate a novel plant immune pathway. Nature. 2015;521:213–6.
32. Mumm K, Ainsaar K, Kasvandik S, Tenson T, Hõrak R. Responses of *Pseudomonas putida* to zinc excess determined at the proteome level: pathways dependent and independent of ColRS. J Proteome Res. 2016;15:4349–68.
33. Jacobs MA, Alwood A, Thaipisuttikul I, Spencer D, Haugen E, Ernst S, et al. Comprehensive transposon mutant library of *Pseudomonas aeruginosa*. Proc Natl Acad Sci. 2003;100:14339–44.
34. Li J, Hu S, Jian W, Xie C, Yang X. Plant antimicrobial peptides: structures, functions, and applications. Bot Stud. 2021;62:5.
35. Badal D, Jayarani AV, Kollaran MA, Kumar A, Singh V. *Pseudomonas aeruginosa* bioflm formation on endotracheal tubes requires multiple two-component systems. J Med Microbiol. 2020;69:906–19.
36. Ansari FA, Ahmad I, Ansari FA, Ahmad I. Biofilm development, plant growth promoting traits and rhizosphere colonization by *Pseudomonas entomophila* FAP1: a promising PGPR. Adv Microbiol. 2018;8:235–51.
37. McBride MB, Blasiak JJ. Zinc and copper solubility as a function of pH in an acid soil. Soil Sci Soc Am J. 1979;43:866–70.
38. Kampfenkel K, Van Montagu M, Inze D. Effects of iron excess on *Nicotiana plumbaginifolia* plants (implications to oxidative stress). Plant Physiol. 1995;107:725–35.
39. Reardon TF, Allen DG. Iron injections in mice increase skeletal muscle iron content, induce oxidative stress and reduce exercise performance. Exp Physiol. 2009;94:720–30.
40. Chandrangsu P, Rensing C, Helmann JD. Metal homeostasis and resistance in bacteria. Nat Rev Microbiol. 2017;15:338–50.
41. Yao J, Rock CO. Phosphatidic acid synthesis in bacteria. Biochim Biophys Acta Mol Cell Biol Lipids. 2013;1831:495–502.
42. Zhang YM, Rock CO. Membrane lipid homeostasis in bacteria. Nat Rev Microbiol. 2008;6:222–33.
43. Hinchliffe P, Yang QE, Portal E, Young T, Li H, Tooke CL, et al. Insights into the mechanistic basis of plasmid-mediated colistin resistance from crystal structures of the catalytic domain of MCR-1. Sci Rep. 2017;7:39392.

44. Perez JC, Shin D, Zwir I, Latifi T, Hadley TJ, Groisman EA. Evolution of a bacterial regulon controlling virulence and Mg^{2+} homeostasis. PLOS Genet. 2009;5:e1000428.
45. Hmelo LR, Borlee BR, Almblad H, Love ME, Randall TE, Tseng BS, et al. Precision-engineering the *Pseudomonas aeruginosa* genome with two-step allelic exchange. Nat Protoc. 2015;10:1820–41.
46. Cheng K, Smyth RL, Govan JRW, Doherty C, Winstanley C, Denning N, et al. Spread of β-lactam-resistant *Pseudomonas aeruginosa* in a cystic fibrosis clinic. Lancet. 1996;348:639–42.
47. Kovach ME, Elzer PH, Steven Hill D, Robertson GT, Farris MA, Roop RM, et al. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. Gene. 1995;166:175–6.
48. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR. Site-directed mutagenesis by overlap extension using the polymerase chain reaction. Gene. 1989;77:51–59.
49. Rietsch A, Vallet-Gely I, Dove SL, Mekalanos JJ. ExsE, a secreted regulator of type III secretion genes in *Pseudomonas aeruginosa*. Proc Natl Acad Sci. 2005;102:8006–11.
50. Bloemberg GV, O'toole GA, Lugtenberg BJJ, Kolter R. Green fluorescent protein as a marker for *Pseudomonas* spp. Appl Environ Microbiol. 1997;63:4543–51.
51. Smith AW, Iglewski BH. Transformation of *Pseudomonas aeruginosa* by electroporation. Nucleic Acids Res. 1989;17:10509.
52. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant. 1962;15:473–97.
53. Andrews S. FASTQC: a quality control tool for high throughput sequence data. 2010. Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.
54. Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. Salmon provides fast and bias-aware quantification of transcript expression. Nat Methods. 2017;14:417–9.
55. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15:550.
56. Zhu A, Ibrahim JG, Love MI. Heavy-Tailed prior distributions for sequence count data: removing the noise and preserving large differences. Bioinformatics. 2019;35:2084–92.
57. Beskrovnaya P, Melnyk RA, Liu Z, Liu Y, Higgins MA, Song Y, et al. Comparative genomics identified a genetic locus in plant-associated *Pseudomonas* spp. that is necessary for induced systemic susceptibility. mBio. 2020;11:1–14.
58. Götz S, García-Gómez JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, et al. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Res. 2008;36:3420–35.
59. Grote S. GOfuncR: gene ontology enrichment using FUNC. R package version 1.10.0. 2020. https://doi.org/10.18129/B9.bioc.GOfuncR.
60. Haney EF, Trimble MJ, Hancock REW. Microtiter plate assays to assess antibiofilm activity against bacteria. Nat Protoc. 2021;16:2615–32.
61. Palmer KL, Aye LM, Whiteley M. Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. J Bacteriol. 2007;189:8079–87.

ACKNOWLEDGEMENTS

This work was supported by a CIHR Grant (PJT - 169051), NSERC Discovery Grant (NSERC-RGPIN-2016-04121), and a Canada Research Chair salary award to CHH. REWH was funded by a CIHR foundation grant (FDN-154287) and received salary support from a Canada Research Chair and UBC Killam Professorship. YZ was supported by an NSERC CGS-M and CLW was supported by an NSERC CGS-D award. DT is supported by an NSF postdoctoral fellowship in Biology (IOS-2010946). MA is supported by a Vanier graduate scholarship. DP was supported by a fellowship from the Michael Smith Foundation for Health Research. We thank Drs. Fred Ausubel and Alina Gutu for conversations that lead to the conception of this project.

AUTHOR CONTRIBUTIONS

Conceptualization, CW, YZ, DP, REWH, and CHH; Methodology, CW and JZ; Formal Analysis, CW, YZ, MA, CDH, MED, AW, DP, and CHH; Investigation, CW, YZ, MA, CDH, MAD, DT, MED, and DP; Resources, REWH and CHH; Data Curation, CW, YZ, AW, and CHH; Writing – Original draft, CW, JZ, and CHH; Writing – Reviewing and Editing, all; Visualization, CW, JZ, MA, CDH, DT, DP, and CHH; Supervision, CHH, DP, and REWH; Funding Acquisition, CHH, and REWH.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41396-022-01343-3.

Correspondence and requests for materials should be addressed to Cara H. Haney.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.