PrbP modulates biofilm formation in *Liberibacter crescens*

Lei Pan[1], Christopher L. Gardner[1], Reagan Beliakoff[1], Danilo Da Silva[1], Ran Zuo[1], Fernando A. Pagliai[1], Kaylie Padgett-Pagliai[1], Marcelo L. Merli[1], Erol Bahadiroglu[1], Claudio F. Gonzalez[1] and Graciela L. Lorca[1]*

[1] Microbiology and Cell Science Department, Genetics Institute, Institute of Food and Agricultural Science, University of Florida, Gainesville, Florida, United States

* Corresponding author: E-mail: glorca@ufl.edu; Phone: 352-273-8090; Fax: 352-273-8284

Keywords: PrbP, transcriptional regulatory protein, *Liberibacter crescens*, biofilm

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/1462-2920.15740

Originality-Significance Statement

Liberibacter asiaticus is the major causative agent of the devastating citrus disease Huanglongbing (HLB). Due to the inability to culture this pathogen, the essential mechanisms used for survival, adaptation and persistence remain largely unknown. In this study, we investigated the regulatory network of PrbP, an important transcription factor, in the model species *L. crescens*. Our results suggest PrbP is a global regulator involved in biofilm formation and numerous cellular processes that are critical for the survival and persistence of *L. asiaticus*. Biofilm formation has long been associated with pathogenesis and persistence of bacteria, and recent studies have reported biofilm formation in *L. asiaticus* and *L. solanacearum*. The discovery that PrbP modulates biofilm formation in the surrogate species *L. crescens* greatly contribute to understandings into the regulatory mechanisms and dynamics of biofilm formation in several pathogenic *Liberibacter* spp.

Summary

In *Liberibacter asiaticus*, PrbP is a transcriptional regulatory protein involved in survival and persistence during host infection. Tolfenamic acid was previously found to inhibit interactions between PrbP and the promotor region of *rplK*, resulting in reduced survival of *L. asiaticus* in the citrus host. In this study, we performed transcriptome analyses to elucidate the PrbP regulon in *L. crescens*, as it is phylogenetically the closest related species to *L. asiaticus* that can be grown in laboratory conditions. Chemical inhibition of PrbP with tolfenamic acid revealed that PrbP is involved in the regulation of diverse cellular processes, including stress response, cell motility, cell cycle and biofilm formation. *In vitro* DNA binding and bacterial two hybrid assays also suggested that PrbP is a global regulator of multiple transcription factors (RpoH, VisN, PleD, MucR, MocR and CtrA) at both transcriptional and/or post-transcriptional levels. Sub-lethal concentrations of tolfenamic acid significantly reduced the attachment of *L. crescens* during biofilm formation and decreased long-term persistence in biofilm structures. Overall, our findings show the importance of PrbP in regulating diverse biological processes through direct and indirect interactions with other transcriptional regulators in *L. crescens*.

Introduction

Huanglongbing (HLB) or citrus greening has plagued the citrus industry for over a century. While its emergence in the Americas (Florida and Brazil) was not until around 2004 (Teixeira, 2005; Bové, 2006), the pathogen quickly spread across the United States and South America and is now considered the most devastating citrus disease in existence (Wang *et al.*, 2017). The causative agents are alpha-proteobacterium members of the *Liberibacter* genera. The inability to culture the main culprit *L. asiaticus* in axenic conditions has severely hampered progress towards understanding the physiology and pathogenesis of this bacterium (Davis *et al.*, 2008; J. R. Fagen *et al.*, 2014; Jain *et al.*, 2019). In an effort to identify bacterial components involved in pathogenesis, the genomic sequence of *L. asiaticus* has been analyzed extensively (Duan *et al.*, 2009; Jennie R. Fagen *et al.*, 2014). Unexpectedly, early *in silico* analysis of the *L. asiaticus* genome did not reveal the presence of genes traditionally associated with virulence (e.g., type II, III, and IV secretion systems and effectors) (Duan *et al.*, 2009). While these canonical virulence factors are frequently used as therapeutic targets in drug design, the absence of them in *L. asiaticus* led to the use of alternative strategies. These include the heterologous expression of *L. asiaticus* proteins, and protein purification, followed by biochemical characterization, studies in phylogenetically related model microorganisms, the transient pathogen's gene expression in alternative hosts as well as the analysis of the citrus host responses during HLB infection. However, most of these studies have only partially examined the differentially expressed genes in *L. asiaticus* during infection (Nwugo *et al.*, 2013; Yan *et al.*, 2013; Zheng and Zhao, 2013; Jain *et al.*, 2015; Pitino *et al.*, 2016; Prasad *et al.*, 2016; Loto *et al.*, 2017). *L. asiaticus* has a reduced genome size (1.23 Mb), which is consistent with other intracellular bacterial species (Moran, 2002; Duan *et al.*, 2009; McCutcheon, 2010). Nonetheless, this pathogen has evolved to survive in multiple hosts, and has rapidly adapted to significant environmental changes (such as sucrose concentrations) during the transition from psyllid vector to citrus host (Pagliai *et al.*, 2014; Wang *et al.*, 2017; Pan *et al.*, 2019). Taken together, the identification of the regulatory network involved in these adaptations, is an essential step in developing a viable treatment for HLB.

PrbP is one of only eleven transcription factors used to regulate gene expression in *L. asiaticus* (Yan *et al.*, 2013; Pagliai *et al.*, 2014; Gardner *et al.*, 2016). PrbP belongs to the

CarD_CdnL_TRCF family; most members of this family regulate gene expression through interactions with RNA polymerase and DNA promoter regions (Garcia-Moreno *et al.*, 2010; Weiss *et al.*, 2012; Bae *et al.*, 2015; Gardner *et al.*, 2016). CarD and CdnL are homologs of PrbP that have been implicated in the pathogenesis of *Mycobacterium* and *Myxococcus*, respectively (Stallings *et al.*, 2009; Garcia-Moreno *et al.*, 2010). Similarly, LtpA from *Borrelia burgdorferi* has been shown to be important for the enzootic cycle of this pathogen (Chen *et al.*, 2018). In *L. asiaticus,* PrbP modulates gene expression via interactions with RNA polymerase and a specific sequence on the promoter region of the ribosomal gene *rplK*. PrbP is post-translationally regulated through interaction with the ferredoxin-like protein FerR, and interactions between PrbP and FerR were found to increase the transcriptional activity of *L. asiaticus* RNA polymerase in a dose dependent manner. FerR was also found to improve the affinity of PrbP for *rplK, in vitro* (Pan et al., 2019). Using small molecule screening, tolfenamic acid (TA) was identified as a molecule that binds PrbP and disrupts interactions with the *rplK* promoter, *in vitro*. When examined in *L. crescens*, the addition of tolfenamic acid was found to inhibit the planktonic cell growth. In HLB-infected citrus, tolfenamic acid significantly decreased the overall transcriptional activity of *L. asiaticus*, and reduced survival of the pathogen in the citrus host (Gardner *et al.*, 2016, 2020; Pan *et al.*, 2017).

Based on the findings that PrbP can modulate gene expression in *L. asiaticus* through interactions with RpoB and FerR, we examined the possibility that PrbP may function as a global regulator through interactions with other proteins, depending on environmental conditions.

To this end, we performed a transcriptome analysis using tolfenamic acid as a chemical inhibitor of PrbP, in the model organism *L. crescens. L. crescens* shares an overall 77.4% nucleotide identity with *L. asiaticus* and is the only cultivable member of the genus *Liberibacter* (Fagen et al., 2014). Consequently, *L. crescens* is frequently used as a surrogate to analyze the function of *L. asiaticus* proteins (Pagliai *et al.*, 2014; Jain *et al.*, 2019; Naranjo *et al.*, 2019). While *L. crescens* can be propagated under laboratory conditions, the genetic manipulation needed to obtain gene deletions is still a challenging area. Therefore, here, we used tolfenamic acid to chemically inactivate PrbP and performed a combination of transcriptome analyses and *in vivo* protein/protein interaction methods to identify the regulatory network of PrbP in *L. crescens*.

In vivo chemical inhibition of PrbP using tolfenamic acid induced transcriptomic profile changes in *L. crescens,* indicating that PrbP is involved in regulating a diversity of cellular processes, including stress responses, cell motility, cell cycle, and biofilm formation. *In vitro* DNA binding and bacterial two hybrid assays suggest PrbP interacts with multiple transcription factors involved in biofilm formation. It was found that culturing cells in presence of tolfenamic acid at sublethal concentrations significantly reduced the attachment of *L. crescens* during biofilm formation as well as its long-term survival.

Results

Inhibition of PrbP activity results in pleiotropic changes in the gene expression profile in *L. crescens* BT-1

PrbP is highly conserved in all *Liberibacter species*, with PrbP from *L. crescens* and *L. asiaticus* sharing 82.84% sequence identity. We have previously shown that the residues involved in TA binding are also conserved among these two species (Pan et al., 2017). To identify the physiological functions that are regulated by PrbP in *Liberibacter,* we used tolfenamic acid to chemically inactivate PrbP activity and analyzed the resulting changes in gene expression. The MIC for tolfenamic acid is 70 μM in *L. crescens* cells (Gardner *et al.*, 2016). For the RNA-seq, *L. crescens* was cultured in presence or absence of non-lethal concentrations (10 μM, Supplementary Figure 1) of tolfenamic acid, and total RNA was subsequently extracted for sequencing.

169 differentially expressed genes were identified following treatment with tolfenamic acid *(Padj* <0.05). Among the differentially expressed genes, 34 were up-regulated and 135 were down-regulated. Overall, the changes in gene expression ranged from -2.15 to 1.0 of log2 fold change (Supplementary Table 3). These observations are consistent with previous reports where it was described that PrbP homologs can either activate or repress gene expression (Gardner *et al.*, 2016; Pan *et al.*, 2017; Zhu *et al.*, 2019). The differentially expressed genes were categorized into functional groups following the Cluster of Orthologues Genes (COG) (**Figure 1**). Functional categorization showed the differentially expressed genes were involved in various cell processes, including transcription and translation machinery, as well as amino acid, nucleotide, lipid, and inorganic iron transport and metabolism (Supplementary Table 3). These results are consistent

previous reports describing genes modulated by other PrbP homologs in *Mycobacterium* species (Stallings *et al.*, 2009; Chen *et al.*, 2018).

Numerous genes involved in general stress responses, biogenesis of extracellular structures, and cell cycle were also significantly affected by inhibiting of PrbP activity. Notably, the expression of several genes encoding heat shock proteins (*B488_12030, dnaK; B488_12040, dnaJ; B488_02560, groEL; B488_02550, groES*), pilus proteins (*B488_06650, flp/pilA*; *B488_12830, cpaD*), and cell division proteins (*B488_05970, ftsZ*; *B488_07460, zapE*; *B488_09690, popZ*) showed significant changes in expression (see Supplementary Table 3 for complete list of differentially expressed genes). Noteworthy, the expression of *rpoH, visN, pleD, mucR, mocR,* and *ctrA*, the transcription factors associated with regulating these cellular processes, was also modified in response to the inhibition of PrbP (**Table 1**). Interestingly, the expression of *prbP* was found to decrease in presence of tolfenamic acid. Our previous studies *in planta* indicated the expression of PrbP is not autoregulated in *L. asiaticus,* suggesting that one of the general transcription factors affected by PrbP (as observed in the liquid cultures) may be involved in the regulation of the expression of PrbP or that PrbP is autoregulated in *L. crescens*.

The changes in expression of these transcriptional regulators were confirmed by qRT-PCR in independently grown cultures (**Table 1**). It was found that while *rpoD, visR,* and *mocR,* were significantly decreased upon TA treatment, or showed similar trends (*mucR, prbP, ctrA* and *rpoH*, although not statistically significant) to the results obtained in the RNAseq, the induction in expression observed in *pleD* and *visN* was not observed.

Homologs of VisNR (*B488_01790* and *B488_01800*, respectively) from *L. asiaticus* were recently shown to regulate motility and expression of the pilus gene *flp*3 *in vitro* and using *Agrobacterium tumefaciens* as a surrogate system (Andrade and Wang, 2019). While no functional studies have been done for the other transcription factors in the *Liberibacter* genus, their regulatory roles can be predicted from other microorganisms. The alternative sigma factor RpoH (*B488_11710*) is known to control the expression of heat shock genes in gram-negative bacteria. PleD (*B488_00790*) is a two-component response regulator containing a diguanylate cyclase domain for turnover of cyclic-di-guanosine monophosphate (C-di-GMP), a signaling molecule that

coordinates motility, cell cycle, and biofilm formation (Sisti *et al.*, 2013; Valentini and Filloux, 2016; Jenal *et al.*, 2017; Kharadi and Sundin, 2019). MucR (*B488_00360*) has been proposed to be involved in the production of exopolysaccharides and cell motility (Bertram-Drogatz *et al.*, 1998; Bahlawane, Baumgarth, *et al.*, 2008; Bahlawane, McIntosh, *et al.*, 2008). MocR (*B488_01350*) regulates a variety of cellular process including pyridoxal phosphate (PLP) biosynthesis, amino acid metabolism, peptidoglycan synthesis, motility, and biofilm formation (Jochmann *et al.*, 2011; Takenaka *et al.*, 2015; Taw *et al.*, 2015; Tramonti *et al.*, 2018). CtrA (*B488_10320*) is a global response regulator for bacterial cell cycle events such as pili and flagella biogenesis and cell division (Quon *et al.*, 1996; Kelly *et al.*, 1998; Reisenauer *et al.*, 1999; Skerker and Shapiro, 2000; Spencer *et al.*, 2009). To determine if PrbP may act as a global transcription factor by modulating the expression and/or activity of other transcription factors in *L. crescens* sequence analyses followed by EMSA analyses were performed.

Analysis of the promotor region of the transcription factors (*rpoH, rpoD, prbP, visN, pleD, mucR, mocR,* and *ctrA*) revealed that the specific DNA binding sequence in *rplK* that was previously identified for PrbP in *L. asiaticus* has replacements in critical nucleotides required for PrbP binding (Supplementary Figure 2**)** (Gardner *et al.*, 2016). While the binding sequence for PrbP may differ between *L. crescens* and *L. asiaticus*, it is possible that PrbP binds to additional sequences that are yet to be identified. To determine if PrbP from *L. crescens* is able to bind the promoter regions of these transcription factors, electrophoresis mobility shift assays (EMSAs) were performed. It was found that $PrbP_{Lcr}$ binds to the promoter region of *mucR* (P*mucR* -255 to +54 using the ATG of *mucR* as a reference), *pleD* (P*pleD* -229 to + 53), *mocR* (P*mocR* -239 to +53), *prbP* (P*prbP* -235 to +54), *visN* (P*visN* -311 to +29), *ctrA* (P*ctrA* -255 to +54), *rpoH* (P*rpoH* -258 to +53), in a concentration dependent manner (0-10 μM) (Figure 2). PrbP binding to *rpoD* (P*rpoD* -211 to +56) and *rplK* (P*rplK* -192 to +83) were used as a positive controls while binding to *hsdR* was used as a negative control (**Figure 2**). The specificity of the binding was confirmed by adding unlabeled probes at increasing concentrations (Supplementary Figure 3). These results suggest that PrbP interacts directly with the promotor regions of *ctrA, rpoH, prbP, mucR, mocR*, *pleD*, and *visN* to regulate gene expression.

PrbP interacts with CtrA, PleD and MucR

In addition to the direct modulation of gene expression through interactions with promoter regions, PrbP may also regulate the expression of numerous genes by interacting with local regulators. We tested the later hypothesis using a β-galactosidase reconstitution based bacterial two-hybrid assay. The selected proteins were fused to two non-functional but complementing β-galactosidase truncations (Δα and Δω). The β-galactosidase activity driven by the protein−protein recognition between both non-β-galactosidase parts of the chimeras allowed analysis of protein-protein interactions (Borloo *et al.*, 2007; Pan *et al.*, 2019). The sequences encoding for RpoH, PleD, VisN, VisR, CtrA, MocR, MucR and PrbP were cloned and fused to the β-galactosidase truncations Δα and Δω on plasmids pB2HΔα and pB2HΔω, respectively. The recombinant plasmids were transformed in the six possible combinations into a β-galactosidase deficient strain (*E. coli* MC1061) and used as reporter strains (Supplementary Table 1). Based on our previous studies in *L. asiaticus* (Pan *et al.*, 2019), FerR and RpoB were included as positive controls.

Higher β-galactosidase activity was observed in constructs 2HB27 (carrying pB2HΔα*_prbP*$_{Lcr}$ and pB2HΔω*_mucR*$_{Lcr}$; **Figure 3A**), 2HB31 (carrying pB2HΔα*_prbP*$_{Lcr}$ and pB2HΔω*_pleD*$_{Lcr}$; **Figure 3B**), and 2HB43 (carrying pB2HΔα*_prbP*$_{Lcr}$ and pB2HΔω*_ctrA*$_{Lcr}$; **Figure 3E**), when compared to the control strains. The strains used to examine PrbP interactions with MocR, VisN and RpoH showed no significant changes in β-galactosidase activity when compared to the control strains (**Figure 3C, 3D, 3F)**. Interestingly, while interactions between PrbP and FerR in *L. crescens* were observed as expected in strain 2HB50 (carrying pB2HΔα*_prbP*$_{Lcr}$ and pB2HΔω*_ferR*$_{Lcr}$; **Figure 3G**) interactions between PrbP and RpoB were not observed (**Figure 3H**). In summary, the results of the two-hybrid assays support the direct interaction of PrbP with PleD, CtrA and MucR in *L. crescens*. When combined with the ability for PrbP to bind the promoter regions of several transcription factors, the protein-protein interactions observed with PrbP suggests that PrbP behaves as a global regulator to coordinate diverse cellular processes at transcriptional and post-transcriptional levels.

The expression of PrbP, MucR, MocR, VisN, CtrA, PleD and RpoH shifts under biofilm-forming conditions

Based on our results, a common denominator among the transcription factors regulated PrbP is their predicted role in biofilm formation. In *Liberibacter,* the VisNR system has been implicated

in modulation of the adherence-pilus (Tad) locus (Andrade and Wang, 2019), while in other microorganisms PleCD and CtrA have been implicated in the regulation of pilus and flagella biogenesis, as well as biofilm formation (Quon *et al.*, 1996; Kelly *et al.*, 1998; Reisenauer *et al.*, 1999; Skerker and Shapiro, 2000; Spencer *et al.*, 2009; Sisti *et al.*, 2013; Valentini and Filloux, 2016; Kharadi and Sundin, 2019).

Since biofilm structures have been visualized in *L. asiaticus* and *L. solanacearum* and may play an important role in the pathogenesis of *L. asiaticus* (Cicero *et al.*, 2016; Ha *et al.*, 2019), the role of PrbP on the expression of *rpoH*, *pleD, visN*, *ctrA*, *mocR*, *mucR* and *prbP* during biofilm forming conditions was explored. *L. crescens* has recently been shown to form a biofilm *in vitro* (Naranjo *et al.*, 2019). By removing fetal bovine serum (FBS) from the rich complex media BM7, *L. crescens* cells can attach to surfaces and form aggregates packed in a polysaccharide matrix (bBM7 media, Naranjo et al., 2019). To evaluate the impact of PrbP regulation in biofilm formation, cells were grown to middle exponential phase in standard BM7 and divided into two groups and pelleted. One group was resuspended in standard BM7 and kept as a control while the other was resuspended in biofilm forming media (bBM7).

Following growth in bBM7, expression of *mucR, visN, ctrA, rpoH*, and *prbP* was significantly ($P<0.05$) up-regulated (5.4-fold, 2.5-fold, 4.0-fold, 3.6-fold, and 10.0-fold, respectively), while expression of *pleD* and *mocR* was significantly ($P<0.05$) down-regulated (-2.5-fold and -2.5-fold, respectively), when compared to controls grown in standard BM7 media (**Figure 4A-F**). These results suggest that MucR, VisN, CtrA, RpoH and PrbP are positively involved in the regulation of *L. crescens* biofilm formation.

Inhibition of PrbP affect the expression of genes related to biofilm development in *L. crescens*.

To determine the role of PrbP in the expression of these transcription factors, cells were incubated in presence or absence of sublethal concentrations (10 μM) of tolfenamic acid, under biofilm promoting conditions (growth in bBM7 media). As expected, the expression of each transcription factor was found to revert to levels similar to the BM7 controls, while expression of *prbP* was affected to a much lesser extent (**Figure 4**). Taken together, these results indicate that PrbP has a

direct role in regulating the transcription of these genes in *L. crescens*. In addition, the moderate decrease in *prbP* expression observed in presence of tolfenamic acid (in bBM7), suggest *prbP* expression may be additionally regulated by a transcription factor positively modulated by PrbP (i.e., *mucR, visN, ctrA,* and *rpoH)* as part of a positive feedback loop.

To confirm the role of PrbP on the regulatory network hierarchy, genes predicted to be regulated directly by *mucR, visN, ctrA, rpoH, pleD* or *mocR* were selected, and their expression levels determined. In *Sinorhizobium meliloti*, MucR was shown to inhibit expression of *rem,* an activator of motility genes (Bahlawane, McIntosh, *et al.*, 2008). In *L. crescens*, the expression of *rem* (*B488_09470*) was repressed (-7.8-fold, $P<0.05$) when *mucR* was induced in bBM7; and as expected, the expression of *rem* was abolished by the addition of tolfenamic acid (**Figure 4B**).
In *Caulobacter crescentus*, PleC is a sensor kinase that autophosphorylates to activate the synthesis of C-di-GMP by PleD (Aldridge *et al.*, 2003). Analysis in *L. crescens* revealed that *pleC* (*B488_05230*) expression was downregulated 2.0-fold ($P<0.05$) when grown in bBM7 media (**Figure 4C**). These results are consistent with the changes of *pleD* expression, suggesting that synthesis of c-di-GMP may be altered in bBM7. The expression of *pleC* was also found to recover in presence of tolfenamic acid, as seen with *pleD* (**Figure 4C**).
The expression of the PLP synthase *pdxJ* and D-alanine-D-alanine ligase (*ddl*) are reported to be controlled by MocR in *Corynebacterium glutamicum* and *Brevibacillus brevis*, respectively (Jochmann *et al.*, 2011; Takenaka *et al.*, 2015). Similar to previous reports, it was observed that expression of *pdxJ* (*B488_01370*) and *ddl* (*B488_05940*) was significantly repressed or activated (-4.6-fold and 4.1-fold, respectively, $P<0.05$) following the decrease in *mocR* mRNA levels in bBM7 media. As expected, the modulating effect on *ddl* expression was completely reversed with tolfenamic acid (**Figure 4D**). A similar trend was observed for *pdxJ* expression, but the effect of tolfenamic acid was not statistically significant.

Using *Agrobacterium* as a model, the VisNR regulators from *L. asiaticus* were recently shown to negatively regulate the expression of *flp3,* a predicted subunit of the pili (Andrade and Wang, 2019). As expected, *flp3* (*B488_12920*) expression was negatively regulated (-6.4-fold, $P<0.05$) in bBM7, when compared to BM7, and the expression of *flp3* was found to fully recover in presence of tolfenamic acid (**Figure 4E**). These results are in accordance with up-regulated *visN*

expression in bBM7. While the alteration of *visR* expression in bBM7 was also found to be reversed by tolfenamic acid treatment, the profile of *visR* expression was unexpectedly opposite (-2.4-fold, $P<0.05$) of *visN* expression (**Figure 4E**). Additionally, the predicted type IV pilus components/assembly genes *cpaD* (*B488_12830*) and *tadC/cpaH* (*B488_12790*) did not show significant differences between each treatment group (**Figure 4E**).

CtrA was reported to bind the promoter region of the pilin subunit gene *pilA*, flagellar gene *fliQ,* and chemotaxis gene *motB*. It has also been shown to regulate spatial and temporal pilus and flagellar biogenesis in *Caulobacter crescentus* (Reisenauer *et al.*, 1999; Skerker and Shapiro, 2000; Spencer *et al.*, 2009). In this experiment, expression of the pilin subunit gene *pilA* (*B488_06650*) was significantly up-regulated (13.6-fold, $P<0.05$) in bBM7 media, when compared to BM7, while expression of the flagellar gene *fliQ* (*B488_09400*) and chemotaxis gene *motB* (*B488_09510*) was significantly repressed (-4.3-fold and -5.0-fold, respectively; $P<0.05$) (**Figure 4F**). Expression of *pilA, motB* and *fliQ* was in accordance with *ctrA* in bBM7 and was reverted to BM7 control level by tolfenamic acid treatment as well (**Figure 4F**).
In *Neisseria gonorrhoeae*, the expression of *dnaK* is RpoH-dependent (Laskos *et al.*, 2004). We found that the expression level of *dnaK* was highly induced in bBM7 (13.6-fold $P<0.05$), while it was significantly decreased by treatment with tolfenamic acid, similar to the expression profile of *rpoH* (**Figure 4G**).

Altogether, these results confirm the involvement of PrbP in the transcriptional control of *mucR, visN, ctrA, rpoH, pleD* and *mocR* under biofilm-forming condition. In addition, we were able to determine that most of the genes predicted to be under the control of these transcription factors in *S. meliloti, C. crescentus* and *C. glutamicum* have a similar mechanism of regulation in *L. crescens*.

Inhibition of PrbP activity significantly reduces early attachment and biofilm formation in *L. crescens*

Based on our results, we hypothesized that PrbP activity is required for *L. crescens* to transition from motile to sessile form. Consequently, a decrease in biofilm formation may be observed upon inhibition of PrbP. To test this hypothesis, *L. crescens* biofilm formation was examined in absence

or presence of increasing concentrations (0-100 μM) of tolfenamic acid. After seven days, the biofilm was quantified with crystal violet staining (Rice *et al.*, 2007; O'Toole, 2010).

The formation of *L. crescens* biofilm was found to decrease in a dose-dependent manner ($P<0.05$) when the culture media was amended with increasing concentrations of tolfenamic acid (Supplementary Figure 4). No significant effect was observed when dimethyl sulfoxide (DMSO), the vehicle control, was used. These results provided preliminary but strong support of the positive role of PrbP activity in *L. crescens* biofilm formation.

In order to determine the impact of chemical inhibition of PrbP on the viability of *L. crescens*, a live/dead staining method was used to visualize *L. crescens* biofilm under confocal laser scanning microscope (CLSM). TA at 10 and 25 μM had a similar non-lethal effect (Supplementary Figure 1), while concentrations over 50 μM reduced the amount of biofilm significantly (Supplementary Figure 4). Based on these results, the concentration of tolfenamic acid was maintained at a sublethal concentration (25 μM) on the treatment group. Z-series of the different groups were obtained for the quantification and reconstruction of 2D and 3D views of the biofilms. At seven days post inoculation (7 dpi), the untreated cells and vehicle controls showed predominantly live cells packed in biofilm structures with similar biomass (3.11±0.59 and 3.32±0.52 $\mu m^3/\mu m^2$, respectively), mean thickness (8.53±2.51 and 9.061±3.07 μm, respectively), and maximum thickness (14.67±1.75 and 14.83±1.26, respectively). Cells that were treated with tolfenamic acid, however, showed a significant ($P<0.05$) decrease in biomass (0.53±0.11 $\mu m^3/\mu m^2$), mean thickness (1.53±0.18 μm), and maximum thickness (3.50±0.87 μm). At 7 dpi, the biomass of the tolfenamic acid treated group was primarily composed of dead cells, with a negligible amount of live cells (0.056±0.03 $\mu m^3/\mu m^2$) (**Figure 5D**). The effects of tolfenamic acid observed at 7 dpi indicate that chemical inhibition of PrbP activity can reduce biofilm formation and decrease long-term survival of *L. crescens*.

To elucidate the mechanism resulting in reduced biofilm formation and cell death following growth in presence of tolfenamic acid, the biofilm formation was visualized and quantified after 1-, 2-, and 4-days post inoculation (1 dpi, 2 dpi, and 4 dpi, respectively). Cells in the bBM7 and vehicle control groups at 1 dpi showed primarily live cells, in loose structures with similar biomass

(0.55±0.23 and 0.59±0.19 $\mu m^3/\mu m^2$, respectively), mean thickness (2.72±0.66 and 2.74±1.05 μm, respectively) and maximum thickness (8.38±1.10 and 8.125±1.11μm, respectively), while the tolfenamic acid treatment group showed significant (P<0.05) reductions in both biomass (0.13±0.04 $\mu m^3/\mu m^2$) and maximum thickness (4.38±0.25 μm) (**Figure 5A**). The mean thickness at 1 dpi, however, was not significantly different between treatment groups.

At 2 dpi, cells in bBM7 and the vehicle control group showed mostly live cells in more compact structures, with similar biomass (1.15±0.25 and 0.95±0.69 $\mu m^3/\mu m^2$, respectively), mean thickness (5.71±1.18 and 4.43±1.672 μm, respectively) and maximum thickness (11.5±1.47 and 11±1.00, respectively). Meanwhile, tolfenamic acid significantly (P< at least 0.05) decreased all three parameters (0.12±0.01 $\mu m^3/\mu m^2$, 1.61±0.38 μm, 4.63±1.44 μm, respectively) (**Figure 5B**). The finding that the tolfenamic acid treatment groups consisted primarily of live cells at 1 and 2 dpi (**Figure 5A-B**), suggests that the decrease in biofilm structure and compactness were likely caused by a decrease in cell attachment.

Similar to observations at 1 and 2 dpi, cells grown in bBM7 and in the vehicle control groups had similar biofilm structures at 4 dpi with primarily live cells. In contrast, the tolfenamic acid treated group showed reductions in mean thickness and maximum thickness (0.49±0.46 and 0.59±0.50 $\mu m^3/\mu m^2$, respectively), and the biomass contained an equal distribution of live and dead cells (**Figure 5C**). These results support the hypothesis that the chemical inhibition of PrbP has a dual effect, it affects cell attachment as well as cell viability in the biofilm.

To evaluate whether the initial attachment of the cells can be further decreased, *L. crescens* was pretreated with tolfenamic acid (25 μM) for 18 hrs in bBM7, prior to inoculating plates for biofilm formation. Biofilm formation was quantified at 1 dpi and 7 dpi. No significant differences were observed between the pretreated and non-pretreated groups (Supplementary Figure 5). These results indicate that 24 h (1 dpi) is sufficient for *L. crescens* to adapt and adjust its gene expression mitigating the effect of the pretreatment.

Together, the CLSM with live/dead staining provided evidence of the role of PrbP activity in facilitating early cell attachment to a static surface, and subsequent biofilm formation.

Discussion

In this study, we elucidated the regulation network of PrbP in *L. crescens.* Using tolfenamic acid as a chemical inhibitor of PrbP, we were able to identify the modulation of a large array of genes when PrbP activity was undermined. Noteworthy, we found that PrbP also modulates the expression of several transcriptional regulators (*mucR*, *visN*, *ctrA*, *rpoH, pleD* and *mocR*) at the level of transcription, and potentially post-translationally through protein-protein interactions (CtrA, MucR and PleD; **Figure 6**).

Interactions between the PrbP homolog (CarD) and RpoB, in the RNA polymerase complex, has been structurally and mechanistically elucidated (Bae *et al.*, 2015; Davis *et al.*, 2015; Rammohan *et al.*, 2015). Similarly, we showed that PrbP from *L. asiaticus* interacts with RpoB to improve transcription initiation *in vitro* (Gardner *et al.*, 2016; Pan *et al.*, 2017). Moreover, these interactions can be further modulated through direct interactions with FerR, a ferredoxin-like protein (Pan et al., 2019). In *M. tuberculosis*, CarD has also been shown to cooperatively stabilize the open complex formation with RbpA, however, they do so through independent interactions with the transcription complex (Rammohan *et al.*, 2016; Jensen *et al.*, 2019). In contrast, in *L. crescens*, PrbP is able to modulate the expression of a large array of genes through direct interactions with transcription factors CtrA, MucR and PleD. This novel mechanism of regulation adds a new layer of control to the complex regulatory mechanisms identified in *Liberibacter*.

PleD, CtrA and MucR are involved in the regulation of stress responses, cell cycle, exopolysaccharide synthesis, motility, and biofilm formation in various bacterial systems. To evaluate the contribution of PrbP to the networks regulated by these transcription factors, we studied the impact of PrbP inhibition under biofilm-forming conditions, where a shift in the mRNA expression profile of PleD, CtrA, RpoH, MucR, MocR and VisN was observed. Homologs of these proteins have been reported to be involved in flagellar and exopolysaccharide biogenesis. MucR regulates cell motility and the production of exopolysaccharides through modulation of rem expression, in addition to regulation by ExpR (Bertram-Drogatz *et al.*, 1998; Bahlawane, Baumgarth, *et al.*, 2008; Bahlawane, McIntosh, *et al.*, 2008). While MocR is known for regulating

PLP-dependent metabolism (*pdxST*, *ddl*) (Jochmann *et al.*, 2011; Takenaka *et al.*, 2015), it has also been proposed to impact motility and biofilm formation (Taw *et al.*, 2015).

RpoH was first recognized for its role in regulating the expression of heat shock genes, however, RpoH homologs are now understood to be induced by a variety of stressors, and their role in general stress response, virulence and biofilm formation is well documented (Bianchi and Baneyx, 1999; Delory *et al.*, 2006; De Lucena *et al.*, 2010; Chu *et al.*, 2018). To confirm the role of PrbP in modulating *pleD, ctrA, rpoH, mucR, mocR* and *visN* expression, we examined the expression profile of genes predicted to be under their control. Overall the data indicate a direct role of PrbP in controlling cellular processes such as flagellar and pili biogenesis, motility, cell cycle and general stress responses. Interestingly, the expression of *pleCD* was down-regulated under biofilm-forming condition, indicating that c-di-GMP synthesis maybe reduced under these conditions. In general, the synthesis of c-di-GMP is positively correlated with biofilm formation in *Vibrio cholerae*, *Pseudomonas aeruginosa, Escherichia coli* and *Salmonella enterica* serovar Typhimurium (Simm *et al.*, 2004; Jones *et al.*, 2015). However, a recent report in the plant pathogen *Erwinia amylovora* suggested that high levels of c-di-GMP lead to increased cell-cell interactions, while decreasing cell-surface attachment (Kharadi and Sundin, 2019). Another exception found in *L. crescens* was that *visN* and *visR* do not seem to express as a single transcript. In *L. crescens,* the mRNA level of *visN* was induced under biofilm forming conditions, however, the expression of *visR* was found to decrease. In contrast, *visN* and *visR* are expressed constitutively in *Sinorhizobium*, and the heterodimer conformation of the proteins (VisN-VisR) is an activator for the expression of flagellar genes (Sourjik *et al.*, 2000). A similar role for the heterodimer activity was recently reported for VisR from *L. asiaticus* using the *Agrobacterium* surrogate. VisR was shown to interact and increase the stability of VisN however, the native expression levels of VisN and VisR were not determined (Andrade and Wang, 2019).

Cell-surface attachment is the initial step of biofilm formation (Palmer *et al.*, 2007). During this step, the Tad pili machinery have been shown to be important for bacterial adherence to host tissues and surfaces (Tomich *et al.*, 2006, 2007; Motherway *et al.*, 2011; Wairuri *et al.*, 2012; Persat *et al.*, 2014; Sangermani *et al.*, 2019). The genomes of *Liberibacter* have been shown to possess the complete set of genes required to assemble the Tad pili machinery (Andrade and Wang, 2019).

The conservation of Tad pilus genes in the severely reduced genomes of *Liberibacter* suggests that this machinery may be of great significance for *Liberibacter* colonization and persistence; and *L. crescens* has recently been shown to form biofilms *in vitro* (Naranjo et al., 2019). Using this model, we were able to confirm the critical role of PrbP in biofilm formation during the early attachment phase, most likely through down regulation of flagellar biogenesis, upregulation of pilus biogenesis via CtrA and PleD, and through interactions with MucR, which regulates exopolysaccharide synthesis and cell motility. The effects on MocR, may also contribute to the successful transition of cells from motile to sessile state and the subsequent development of biofilm. Following biofilm formation, the viability of *L. crescens* was reduced upon inhibition of PrbP, suggesting an essential role for PrbP in biological processes such as stress response, ribosomal biogenesis and metabolic homeostasis, through modulation of *rpoH* expression. While our previous studies have shown that tolfenamic acid binds to PrbP and consequently changes the function of PrbP i*n vitro* (Gardner et al., 2016, Pan et al., 2017), a limitation of the current study is that non-specific effects of tolfenamic acid on *L. crescens* cannot be ruled out *in vivo*. Further confirmatory studies using *prbP* mutants in *L. crescens* are needed however, PrbP homologs in *Mycobacterium* have been reported to be essential (Stallings et al., 2009).

Overall, our work demonstrates that PrbP is involved in transcriptional and post-transcriptional regulation of several key factors involved in stress responses, cell motility, cell cycle and biofilm formation. Furthermore, the high similarity of PrbP among *Liberibacter spp.* suggests that a similar role of PrbP may exist in *L. asiaticus*.

Experimental Procedures

Strains and Growth Conditions.

Escherichia coli strains were grown at 37 °C under aerobic conditions in Lysogen Broth (LB) or on LB agar plates. *E. coli* DH5α (Invitrogen) was used to maintain and replicate all plasmids. *E. coli* BL2-Rosetta (DE3) (Novagen) was used to overexpress and purify recombinant proteins. *E. coli* MC1061 was used to construct two-hybrid reporter strains. When required, the culture

medium was supplemented with ampicillin (100 µg/ml) and/or chloramphenicol (25 µg/ml). All antibiotics and chemicals were purchased from Sigma-Aldrich.

L. crescens BT-1 was cultured at 28 °C with rotary shaking (180 rpm) in BM7 media (Jain *et al.*, 2015), prepared as follows: 2 g of alpha-ketoglutaric acid, 10 g of *N*-(2-Acetamido)-2-aminoethanesulfonic acid (ACES) and 3.75 g of KOH were dissolved in 550 ml of water (pH 6.9) and sterilized by autoclaving. After cooling to room temperature, the solution was combined with 300 mL of filter sterilized TNM-FH insect medium and 150 ml of fetal bovine serum (FBS). For biofilm experiments, bBM7 was used. The bBM7 media is defined as BM7 without FBS. All bacterial strains and plasmids used in this study are listed in Supplementary Table 1.

DNA Manipulations and Gene Cloning

Chromosomal DNA was isolated using the Qiagen DNeasy Kit, and plasmid extractions were performed with the QIAprep Spin Miniprep Kit (Qiagen). Q5 high-fidelity DNA polymerase master mix (NEB) was used for PCR amplification. PCR products were purified using QIAquick Purification Kit (Qiagen). Standard molecular protocols described in Molecular Cloning (Sambrook *et al.*, 2001) were used to perform PCR, restriction enzyme digestion, construction of recombinant DNA molecules, and cell transformations. To clone and purify *L. crescens* PrbP*, prbP* (B488_01720) was amplified by PCR, using chromosomal DNA from *L. crescens,* and cloned into the p15TV-Lic plasmid as described previously (Gardner *et al.*, 2016). For construction of the bacterial two-hybrid assay, each gene was cloned into plasmids pB2HΔα and pB2HΔω as previously described (Borloo *et al.*, 2007; Gardner *et al.*, 2016). Fusion of the genes was verified by sequencing. *E. coli* MC1061, a β-galactosidase deficient strain, was co-transformed with the recombinant plasmids carrying the fusions. Strains carrying the empty vectors or single protein fusion plasmids were used as controls to determine baseline activity.
All oligomers used in this study are listed in Supplementary Table 2.

Transcriptome Analysis

Transcriptome studies were performed as described previously (Pagliai *et al.*, 2017). Briefly, *L. crescens* BT-1 cells were grown with 10 μM tolfenamic acid (in 0.05% dimethyl sulfoxide) while the control was grown with 0.05% DMSO. *L. crescens* BT-1 cells were collected at OD_{600} = 0.3 (mid-exponential phase) by centrifugation at 8000 rpm, at 4 °C. Total RNA was extracted with the RiboPure-Bacteria (Life Technologies) kit following the manufacturer's protocol. The quality of isolated RNA samples was examined in an Agilent 2100 Bioanalyzer. The rRNA was depleted using the MICROBExpress Bacterial mRNA enrichment kit (Life Technologies) according to the manufacturer's instructions. Single end RNA libraries were prepared using the TruSeq Stranded mRNA Library Prep Kit (Illumina) and followed by sequencing using a HiSeq2500 system. RNA sequencing was performed by the Genome Sciences Facility at Penn State University in Hershey, PA. The assays were performed in duplicates, and approximately 2 million reads were obtained for each library.

The sequencing data were analyzed using a pipeline allocated at the HiPerGator supercomputer, University of Florida. In brief, the raw sequences were modified to trim the Illumina sequencing adapter using Cutadapt, followed by sequence processing using the Sickle tool (Joshi and Fass, 2011) to verify the quality of the samples prior to further analyses. Next, sequences were mapped against the genome of *L. crescens* BT-1 using Bowtie2. tRNA and the remaining rRNA sequences were depleted *in silico*. The mapped mRNA sequences were aligned using Samtools and counted using the HTseq tool. The DESeq2 package was used to compare the abundance of each transcript from cells grown in presence of tolfenamic acid to the transcripts obtained from cells grown with the vehicle control (0.05% DMSO). Genes with a relative fold change ≥ 1.17 and padj values < 0.05 were considered for further analysis.

The pathway analyses of differentially expressed genes was performed using Blast2GO (Conesa *et al.*, 2005). The functional classification was based on the Cluster of Orthologues Genes (COG). The percentage of differentially expressed genes within each COG was calculated as the number of hits from a given category in the RNA-seq experiments, divided by the total number of genes present within that COG in the genome of *L. crescens* BT-1.

β-galactosidase Assay

E. coli MC1061 cultures were grown at 37 °C in LB medium. Expression of the fusion proteins was induced by the addition of 0.5 mM isopropyl-thio-β-D-galactopyranoside (IPTG). Cells were collected at late exponential phase (OD_{600} = 1). Cells were lysed in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol; Miller, 1972). β-galactosidase activity was determined by following the catalytic hydrolysis of chlorophenol red-β-D-galactopyranoside (Sigma-Aldrich). The absorbance at 570 nm was read every minute for 30 min using a Synergy HT 96-well plate reader (BioTek). The β-galactosidase activity was calculated using the slope of the absorbance curve normalized by the sample cell density and expressed as arbitrary units (AU). Each reaction was performed with three biological and technical replicates.

Protein Purification

Purification of PrbP was performed as previously described (Gardner *et al.*, 2016). Briefly, the expression strains were grown in LB media with shaking (200 rpm) at 37°C, to an OD_{600} of 0.6. Expression was induced by addition of 0.5 mM IPTG followed by overnight incubation at 17 °C. The cells were harvested by centrifugation and re-suspended in binding buffer (500 mM NaCl, 5% glycerol, 50 mM HEPES, 5 mM imidazole, pH 7.5) supplemented with EDTA-free Protease Inhibitor (Thermo Fisher Scientific). Immediately before lysing, 0.5 mM of Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) was added to the cell suspension. Cells were lysed by passing through a high-pressure French press cell 3 times, at 1500 psi. The lysates were centrifuged at 17,000 × g, at 4 °C for 45 min; the supernatant was recovered and applied to a nickel affinity column (His60 Ni Superflow Resin, Clontech). The loaded column was washed extensively with binding buffer containing 20 mM imidazole, and the protein were eluted from the column in elution buffer (binding buffer with 250 mM imidazole). The eluted protein was dialyzed overnight at 4 °C against 10 mM HEPES (pH 7.5), 500 mM NaCl, 2.5% glycerol, 0.5 mM TCEP, then aliquoted and stored at −80 °C.

Electrophoresis mobility shift assays

Electrophoresis mobility shift assays (EMSA) were performed as described earlier (Gardner *et al.*, 2016). Briefly, a fragment of promoter region was amplified by PCR using pre-labeled 5'-biotin primers. The reaction mix contained 10 ng of 5'-Biotin labeled DNA probe, 10 mM HEPES (pH 7.5), 250 mM NaCl, 5% glycerol, 12.5 ng/µl of non-specific competitor Poly(dI-dC), and purified PrbP protein (0-10 µM). Following incubation at 37°C for 20 min, the samples were analyzed by electrophoresis using 6% acrylamide-bisacrylamide non-denaturing gels, in ice-cold 0.5X Tris-borate EDTA buffer (TBE) at pH 8.3. Samples were transferred from the polyacrylamide gel to a Hybond-N+ membrane (GE Healthcare) by electro-blotting at 250 mA for 45 min, using a semi-dry transfer blot (Fisher Scientific). The transferred DNA was UV-crosslinked and the biotin-labeled DNA was detected using the Phototope-Star Detection Kit (NEB). Membranes were exposed to Kodak X-ray films. Vehicle controls were included in all assays.

Real-Time Quantitative Reverse Transcriptase PCR (qRT-PCR)

L. crescens BT-1 cells were cultured in 3 ml of BM7 liquid media at 28 °C, with rotary shaking (180 rpm) until OD_{600} reached 0.4 (exponential phase). Cells were then collected by centrifugation (4,000 rpm for 10 min) at 28 °C, and subsequently resuspended in 3 ml of each respective media: BM7, bBM7 (BM7 without FBS), bBM7 with 0.05% DMSO (vehicle control), or bBM7 media with 25 µM tolfenamic acid. Cultures were then incubated with rotary shaking (180 rpm), at 28 °C, for 12 hrs and collected by centrifugation. Total RNA extractions were performed using RiboPure RNA Purification Kit-Bacteria (Life Technologies) following the manufacturer's protocol. cDNAs were synthesized using iScript cDNA Synthesis Kit (BioRad). qRT-PCR assays were performed with biological triplicates and technical duplicates for each sample. PowerUp SYBR Green Master Mix (ThermoFisher Scientific) was used as recommended by the manufacturer and reactions were carried out using a QuantStudio 6 (Life Technologies). Changes in gene expression were determined using the ΔΔCt method, and 16S rRNA was used as the internal control. Primers used for qRT-PCR experiments are described in Supplementary Table 2.

Growth of Biofilm

For growth of biofilm, *L. crescens* BT-1 culture were started from 40 µl of frozen stock in BM7 media. After overnight incubation at 28 °C, with rotary shaking at 180 rpm, 3 ml of fresh BM7 media was inoculated to OD_{600} = 0.02. The cultures were grown under the same conditions until OD_{600} = 0.6 after which the cells were harvested by centrifugation at 4000 rpm for 10 min at 28 °C. The pellet was washed once by suspending in 3 ml of bBM7 media followed by centrifugation as described above. The pellet was then resuspended in bBM7 media supplemented with 0.75 mg/ml of methyl-β-cyclodextrin (mβc) (Naranjo *et al.*, 2019) to an OD_{600} = 0.1. The vehicle control (DMSO) and tolfenamic acid was added to the cell suspension as indicated. The bacterial cell suspension was used to inoculate 48-well Tissue Culture Plates (flat bottom, tissue culture treated, polystyrene; Genesee Scientific) or 96-well Special Optics Plates (flat bottom, tissue culture treated, black with ultra-thin clear bottom, polystyrene; Corning) for subsequent assays.

Crystal Violet Staining and Quantification of Biofilm

For crystal violet staining and quantification, growth of *L. crescens* cells and preparation of cell suspension was performed as described above. For each treatment group, six biological replicates were performed. Media alone, bBM7 and BM7 were included as background controls. After inoculation, the plate was sealed with air permeable AeraSeal film (Excel Scientific), covered with low-evaporation lid, and placed with a moisture pack to minimize cross-contamination and evaporation. The plate was incubated at 28 °C for 7 days, prior to staining and quantification.
Staining and quantification of biofilm was performed as previously described (Rice *et al.*, 2007; O'Toole, 2010) with modifications. Briefly, after incubation, culture media was gently removed from wells. The wells were washed by gently adding and removing 1 ml of water. Additionally, 1 ml of methanol was added and removed. The plate was dried in a fume hood for 20 min. The wells were stained with 350 µl of 0.1% crystal violet in water for 15 min at room temperature. The crystal violet stain was removed and wells were washed 2 times with water. After final wash, the plate was dried in a fume hood. The crystal violet stain retained in the wells was solubilized in 300 µl of 30% acetic acid, transferred to a 96-well plate and read at 550 nm using a Synergy HT plate reader (BioTek).

Confocal Laser Scanning Microscopy (CLSM)

Two sets of *L. crescens* cultures were grown as described above for confocal laser scanning microscopy analysis of biofilm. For each well, 200 µl of the cell suspension were inoculated. After inoculation, the plate was sealed with AeraSeal film and incubated at 28 °C for 1-7 days until CLSM observations of biofilm were made as indicated in the results. After incubation, the wells were gently washed twice with 200 µl of 0.85% NaCl solution. The samples were stained using FilmTracer Live/Dead Biofilm Viability Kit for 30 min at room temperature following manufacturer's instructions (Invitrogen). After removing the stain, 200 µl of 0.85% NaCl solution was added for CLSM observation. Biofilm images were collected using a Zeiss LSM800 confocal scanning system with a Plan-Apochromat 20X/0.8 M27 objective. The SYTO-9 and PI fluorophores were exited with an argon laser at 488 nm and 516 nm, respectively, and the emission band-pass filters used for SYTO-9 and PI were 500 nm and 617 nm, respectively. All Z-series were performed with the same parameters used for the control group (untreated *L. crescens* biofilm). Acquisition and processing of images was performed using the ZEN Light (Carl Zeiss, Jena, Germany). Quantification of the biofilms was performed using the COMSTAT2 package. Quantification of biomass and thickness was done using data of at least three Z-stack series from independent biological replicates.

Statistical Analyses

The statistical significance of the qRT-PCR and biofilm quantifications was assessed by the analysis of variance (ANOVA) and a Tukey's HSD post-hoc test; the significance threshold was set at 0.05, and alpha= 0.05 was used for the Tukey's HSD testing.

Data availability

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002; Barrett *et al.*, 2013)and are accessible through GEO Series accession number GSE182163 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc= GSE182163).

Acknowledgements

We would like to thank Dr. Kelly Rice and Mr. Matthew Turner for their technical assistance with biofilm experiments and CLSM microscopy. We express our gratitude to Dr. Ana Conesa for her guidance and access to BLAST2go. We thank Rudy Alvarado from Electron Microscopy Core at the University of Florida Interdisciplinary Center for Biotechnology Research, for his technical support. This work is supported by the Department of Microbiology and Cell Science, the Specialty Crop Research Initiative (Award number 2015-70016-23029 to G.L.L. and C.F.G), the Plant Biotic Interactions Program (Award number 2017-03060 to C.F.G.) from the USDA National Institute of Food and Agriculture http://nifa.usda.gov/. The content is solely the responsibility of the authors and does not necessarily represent the official views of the granting agency.

Conflict of interests

The authors declare no conflict of interests.

FIGURE LEGENDS

Figure 1. Chemical inhibition of PrbP results in pleotropic transcriptional changes in *L. crescens*. Functional classification of the differentially expressed (DE) genes in presence of tolfenamic acid (TA) in *L. crescens* categorized by Cluster of Orthologues Genes (COG). The percentage of differentially expressed genes within each COG was calculated as the number of differentially expressed genes from a given COG divided by the total number of genes predicted within that COG in the genome of *L. crescens BT-1*. Green, up-regulated genes; red, down-regulated genes.

Figure 2. PrbP binds to the promoter region of transcriptional regulators *pleD, visN, mucR, mocR, prbP, rpoH, rpoD* and *ctrA*. EMSAs were performed using biotin labeled promoter regions of *mucR*, *pleD*, *mocR*, *visN*, *ctrA*, *rpoH, rpoD, prbP, rplK, and hsdR* genes incubated with increasing concentrations (0-10 μM) of purified PrbP as indicated in each panel. For each probe, 0 indicates that PrbP was excluded from the reaction mix as a negative control.

Figure 3. Interactions between PrbP and MucR, PleD, CtrA and FerR using a bacterial two-hybrid system. β-galactosidase activity was determined using *E. coli* MC1061 as a reporter strain.

The information of the genetic constructs is shown in Supplementary Table 1. β-galactosidase assays were performed at late exponential phase (OD_{600} = 1). Enzymatic activities are shown as the average arbitrary units (AU) with SD from biological and technical triplicates. Statistical significance was determined as described in Experimental Procedures. *P<0.05; **P< 0.01; ***P<0.001; ****P<0.0001.

Figure 4. Inhibition of PrbP under biofilm forming conditions modulates the expression level of transcriptional regulators and its regulated genes. The expression levels of the transcription regulators and associated genes are shown as follow: **(A)** *prbP* and *rpoD*, **(B)** *mucR*, **(C)** *pleD*, **(D)** *mocR*, **(E)** *visN,* **(F)** *ctrA*, **(G)** *rpoH*. Cells were grown in the following media: BM7, BM7 standard media; bBM7, BM7 media without FBS; bBM7+DMSO, bBM7 + 0.05% DMSO vehicle control; bBM7+TA, bBM7 with 10 μM tolfenamic acid in 0.05% DMSO. mRNA extractions were performed after 12 hours of incubation. The relative expression value of each gene was normalized to the expression levels of gyrase subunit A gene (*gyrA*) with SD from biological triplicates. Statistical significance was determined as described in Experimental Procedures. *P<0.05; **P< 0.01; ***P<0.001; ****P<0.0001.

Figure 5. Inhibition of PrbP activity decreases *L. crescens* attachment during early stage of biofilm formation as well as long-term viability. CLSM observations and quantification of *L. crescens* biofilms was performed in absence or presence (25 μM) of tolfenamic acid. Cell viability was evaluated using Filmtracer Live/Dead stain after **(A)** 1, **(B)** 2, **(C)** 4 or **(D)** 7 days post-inoculation (dpi). Green, live cells; red, dead cells. bBM7, no treatment; bBM7 + DMSO, vehicle control; TA, bBM7 + 25 μM of tolfenamic acid. Representative 2D and 3D views rendered from Z-series of at least three independent biological replicates. Quantification of CLSM observations were performed using ImageJ and Comstat2 package. Statistical significance was determined from at least three independent biological replicates as described in Experimental Procedures. Different letters on top of bars denotes statistical significance of P<0.05.

Figure 6. Proposed model of PrbP regulatory network in *L. crescens*. The regulatory levels are represented where rounded rectangles indicate the encoded proteins. Arrows and bar-ending

arrows indicate activation and repression, respectively. The protein-protein interaction is represented as green line. The transcription regulation is represented as blue line.

Supplementary Figure 1. Effect of increasing concentrations of tolfenamic acid on *L. crescens* growth. *L. crescens* was cultured at 28 ˚C with moderate aeration (150 RPM), in BM7 media, with increasing concentrations (0–100 μM) of tolfenamic acid (TA) as indicated: (x) Control BM7, (◊) BM7 with DMSO 0.05% (vehicle control); (∇) BM7 with 10 μM TA; (Δ) BM7 with 25 μM TA; (□) BM7 with 50 μM TA; (o) BM7 with 100 μM TA. Bacterial growth was determined by optical density at 600 nm (A600), over a period of 5 days.

Supplementary Figure 2. Alignment of the predicted PrbP binding sites in the promoter region of the *mucR*, *pleD*, *mocR*, *visN*, *ctrA*, *rpoH, rpoD,PrbP and rplK* genes. (A) The predicted PrbP binding sequences were obtained by screening the promoter region for sequences with highest similarity to the xAxxxxGGTTxxxxxxxxxAAA PrbP binding site in *L. asiaticus* (Gardner *et al.*, 2016). The boxed nucleotides indicate the crucial positions for PrbP binding, and nucleotides highlighted in yellow indicate mismatched sequence. The sequences were enumerated to indicate the presence of multiple predictions within a single promoter and R indicate that the sequence is on the reverse complementary strand. **(B)** Sequence logo generated from the predicted PrbP binding sites (Crooks *et al.*, 2004).

Supplementary Figure 3. PrbP binds specifically to the promoter regions of the *pleD*, *visN*, mucR, *mocR*, *prbP*, *rpoH*, *rpoD* and *ctrA* transcriptional regulators. (A) For competition experiments, biotin labeled probes for each gene (2 ng) were incubated with 10 μM PrbP and mixed with increasing concentrations (20–2000 ng) of its corresponding unlabeled, double stranded probe as indicated above each panel. For each probe, 0 indicates that PrbP was excluded from the reaction mix, as a negative control.

Supplementary Figure 4. Inhibition of PrbP activity decrease *L. crescens* biofilm formation. Quantification of *L. crescens* biofilm formation was quantified using crystal violet staining. Cells

were inoculated with increasing concentration of tolfenamic acid (TA, 0-100 μM, as indicated in the figure) and biofilm formation was evaluated 7-day post inoculation (dpi). bBM7 and bBM7 + DMSO, were used as no treatment controls. Different letters on top of bars denotes statistical significance of at least $P<0.05$.

Supplementary Figure 5. Pre-treatment of *L. crescens* with TA does not increase the inhibitory effect of TA during biofilm formation. Cells were preincubated in presence or absence of TA (10 μM) for 12 hours and inoculated into microwell plates. CLSM observations and quantification of *L. crescens* biofilms was performed in presence or absence of tolfenamic acid (25 μM). The viability of the cells was evaluated using Filmtracer Live/Dead stain after at **(A)** 1 or **(B)** 7 days post inoculation (dpi). Green, live cells; red, dead cells. bBM7, no treatment; bBM7 + DMSO, vehicle control; TA, bBM7 + 25 μM of tolfenamic acid. TA Pretreated, indicates cultures pretreated with 10 μM TA prior to inoculation. Cells maintained in bBM7 for 12 hours were used as controls. Representative 2D and 3D views rendered from Z-series of at least three independent biological replicates. Quantification of CLSM observations were performed using ImageJ and Comstat2 package. Statistical significance was determined from at least three independent biological replicates as described in Experimental Procedures. Different letters on top of bars denotes statistical significance of at least $P<0.05$.

Table 1. Differentially expressed transcription factors upon PrbP inhibition with tolfenamic acid identified by RNAseq.

Supplementary Table 1. Strains and Plasmids used in this study.

Supplementary Table 2. Primers used in this study.

Supplementary Table 3. Differentially expressed genes upon PrbP inhibition with tolfenamic acid identified by RNAseq.

Table 1. Differentially expressed transcription factors upon PrbP inhibition with tolfenamic acid identified by RNAseq.

Locus tag	Gene name	Gene product	Regulatory function	Relative expression (fold change)	*P*adj value	qRT PCR (Fold change)	*P* value	Predicted PrbP binding site*
B488_0036 0	*mucR*	MucR family transcription regulator	Exopolysaccharide; motility	0.85	0.038	0.87±0.0 9	NS (*P*=0.07)	-176 to -155
B488_0079 0	*pleD*	PleD family two-component response regulator	C-di-GMP levels; surface attachment; motility	1.38	0.001	1.01±0.0 9	NS	-215 to -194; -123 to -102; -87 to -108
B488_0135 0	*mocR*	MocR family transcription regulator	PLP synthesis; peptidoglycan synthesis; motility and biofilm formation; amino acid metabolism	0.81	0.017	0.59±0.0 6	<0.001	-98 to -77
B488_0172 0	*prbP*	CarD family transcription regulator	Stress response; pathogenesis; antibiotic resistance	0.82	<0.00 1	0.78±0.0 4	NS	-127 to -106; -57 to -36; +15 to +36

B488_0179 0	*visN*	LuxR family transcription regulator	Quorum sensing; motility; biofilm formation	2.00	<0.00 1	0.94±0.1 5	NS	-308 to -287; -129 to -108
B488_0180 0	*visR*	LuxR family transcription regulator	Quorum sensing; motility; biofilm formation	1.11	0.486	0.71±0.1 5	0.037	NA
B488_1032 0	*ctrA*	Cell cycle response regulator	Cell cycle control; flagella; chemotaxis;	0.69	<0.00 1	0.80±0.1 9	NS	-39 to -18
B488_1171 0	*rpoH*	Sigma factor 32	Heat shock sigma factor	0.76	<0.00 1	1.04±0.1 3	NS	-152 to -131; -122 to -101; -121 to -143
B488_1335 0	*rpoD*	Sigma factor 70	primary sigma factor	0.74	<0.00 1	0.65±0.1 2	0.002	-204 to -183; -37 to -58

*Relative position from ATG translation start codon; NS, not significant

References

Aldridge, P., Paul, R., Goymer, P., Rainey, P., and Jenal, U. (2003) Role of the GGDEF regulator PleD in polar development of *Caulobacter crescentus*. *Mol Microbiol* **47**: 1695–1708.

Andrade, M. and Wang, N. (2019) The Tad Pilus Apparatus of '*Candidatus* Liberibacter asiaticus' and Its Regulation by VisNR. *Mol Plant-Microbe Interact* **32**: 1175–1187.

Bae, B., Chen, J., Davis, E., Leon, K., Darst, S.A., and Campbell, E.A. (2015) CarD uses a minor groove wedge mechanism to stabilize the RNA polymerase open promoter complex. *Elife* **4**: 1–19.

Bahlawane, C., Baumgarth, B., Serrania, J., Rüberg, S., and Becker, A. (2008) Fine-Tuning of galactoglucan biosynthesis in *Sinorhizobium meliloti* by differential WggR (ExpG)-, PhoB-, and MucR-dependent regulation of two promoters. *J Bacteriol* **190**: 3456–3466.

Bahlawane, C., McIntosh, M., Krol, E., and Becker, A. (2008) *Sinorhizobium meliloti* regulator MucR couples exopolysaccharide synthesis and motility. *Mol Plant-Microbe Interact* **21**: 1498–1509.

Bertram-Drogatz, P.A., Quester, I., Becker, A., and Pühler, A. (1998) The *Sinorhizobium meliloti* MucR protein, which is essential for the production of high-molecular-weight succinoglycan exopolysaccharide, binds to short DNA regions upstream of exoH and exoY. *Mol Gen Genet* **257**: 433–441.

Bianchi, A.A. and Baneyx, F. (1999) Hyperosmotic shock induces the sigma32 and sigmaE stress regulons of *Escherichia coli*. *Mol Microbiol* **34**: 1029–1038.

Borloo, J., De Smet, L., Vergauwen, B., Van Beeumen, J.J., and Devreese, B. (2007) A β-Galactosidase-Based Bacterial Two-Hybrid System To Assess Protein−Protein Interactions in the Correct Cellular Environment. *J Proteome Res* **6**: 2587–2595.

Bové, J.M. (2006) Huanglongbing : A Destructive, Newly-Emerging, Century-Ols Disease of Citrus. *J Plant Pathol* **88**: 7–37.

Chen, T., Xiang, X., Xu, H., Zhang, X., Zhou, B., Yang, Y., et al. (2018) LtpA, a CdnL-type CarD regulator, is important for the enzootic cycle of the Lyme disease pathogen. *Emerg Microbes Infect* **7**: 126.

Chu, E.K., Kilic, O., Cho, H., Groisman, A., and Levchenko, A. (2018) Self-induced mechanical stress can trigger biofilm formation in uropathogenic *Escherichia coli*. *Nat Commun* **9**:.

Cicero, J.M., Fisher, T.W., and Brown, J.K. (2016) Localization of '*Candidatus* Liberibacter solanacearum' and Evidence for Surface Appendages in the Potato Psyllid Vector. *Phytopathology* **106**: 142–154.

Cicero, J.M., Fisher, T.W., Qureshi, J.A., Stansly, P.A., and Brown, J.K. (2017) Colonization and intrusive invasion of potato psyllid by "*Candidatus* Liberibacter solanacearum." *Phytopathology*.

Conesa, A., Götz, S., García-Gómez, J.M., Terol, J., Talón, M., and Robles, M. (2005) Blast2GO: A universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* **21**: 3674–3676.

Davis, E., Chen, J., Leon, K., Darst, S.A., and Campbell, E.A. (2015) Mycobacterial RNA polymerase forms unstable open promoter complexes that are stabilized by CarD. *Nucleic*

Acids Res **43**: 433–45.
Davis, M.J., Mondal, S.N., Chen, H., Rogers, M.E., and Brlansky, R.H. (2008) Co-cultivation of '*Candidatus* Liberibacter asiaticus' with Actinobacteria from Citrus with Huanglongbing. *Plant Dis* **92**: 1547–1550.
Delory, M., Hallez, R., Letesson, J.J., and De Bolle, X. (2006) An RpoH-like heat shock sigma factor is involved in stress response and virulence in *Brucella melitensis* 16M. *J Bacteriol* **188**: 7707–7710.
Duan, Y., Zhou, L., Hall, D.G., Li, W., Doddapaneni, H., Lin, H., et al. (2009) Complete Genome Sequence of Citrus Huanglongbing Bacterium, '*Candidatus* Liberibacter asiaticus' Obtained Through Metagenomics. *Mol Plant-Microbe Interact* **22**: 1011–1020.
Fagen, J. R., Leonard, M.T., Coyle, J.F., McCullough, C.M., Davis-Richardson, A.G., Davis, M.J., and Triplett, E.W. (2014) *Liberibacter crescens* gen. nov., sp. nov., the first cultured member of the genus *Liberibacter*. *Int J Syst Evol Microbiol* **64**: 2461–2466.
Fagen, Jennie R., Leonard, M.T., McCullough, C.M., Edirisinghe, J.N., Henry, C.S., Davis, M.J., and Triplett, E.W. (2014) Comparative Genomics of Cultured and Uncultured Strains Suggests Genes Essential for Free-Living Growth of *Liberibacter*. *PLoS One* **9**: e84469.
Garcia-Moreno, D., Abellon-Ruiz, J., Garcia-Heras, F., Murillo, F.J., Padmanabhan, S., and Elias-Arnanz, M. (2010) CdnL, a member of the large CarD-like family of bacterial proteins, is vital for *Myxococcus xanthus* and differs functionally from the global transcriptional regulator CarD. *Nucleic Acids Res* **38**: 4586–4598.
Gardner, C.L., Pagliai, F.A., Pan, L., Bojilova, L., Torino, M.I., Lorca, G.L., and Gonzalez, C.F. (2016) Drug Repurposing: Tolfenamic Acid Inactivates PrbP, a Transcriptional Accessory Protein in *Liberibacter asiaticus*. *Front Microbiol* **7**: 1630.
Gardner, C.L., da Silva, D.R., Pagliai, F.A., Pan, L., Padgett-Pagliai, K.A., Blaustein, R.A., et al. (2020) Assessment of unconventional antimicrobial compounds for the control of '*Candidatus* Liberibacter asiaticus', the causative agent of citrus greening disease. *Sci Rep*.
Ghanim, M., Fattah-Hosseini, S., Levy, A., and Cilia, M. (2016) Morphological abnormalities and cell death in the Asian citrus psyllid (Diaphorina citri) midgut associated with *Candidatus* Liberibacter asiaticus. *Sci Rep*.
Ha, P.T., He, R., Killiny, N., Brown, J.K., Omsland, A., Gang, D.R., and Beyenal, H. (2019) Host-free biofilm culture of "*Candidatus* Liberibacter asiaticus," the bacterium associated with Huanglongbing. *Biofilm* **1**: 100005.
Jain, M., Cai, L., Fleites, L.A., Munoz-Bodnar, A., Davis, M.J., and Gabriel, D.W. (2019) *Liberibacter crescens* Is a Cultured Surrogate for Functional Genomics of Uncultured Pathogenic '*Candidatus* Liberibacter' spp. and Is Naturally Competent for Transformation . *Phytopathology* PHYTO-04-19-012.
Jain, M., Fleites, L.A., and Gabriel, D.W. (2015) Prophage-Encoded Peroxidase in '*Candidatus* Liberibacter asiaticus' Is a Secreted Effector That Suppresses Plant Defenses. *Mol Plant-Microbe Interact*.
Jenal, U., Reinders, A., and Lori, C. (2017) Cyclic di-GMP: second messenger extraordinaire. *Nat Rev Microbiol* **15**: 271–284.
Jensen, D., Manzano, A.R., Rammohan, J., Stallings, C.L., and Galburt, E.A. (2019) CarD and RbpA modify the kinetics of initial transcription and slow promoter escape of the Mycobacterium tuberculosis RNA polymerase. *Nucleic Acids Res* **47**: 6685–6698.
Jochmann, N., Götker, S., and Tauch, A. (2011) Positive transcriptional control of the pyridoxal phosphate biosynthesis genes pdxST by the MocR-type regulator PdxR of *Corynebacterium*

glutamicum ATCC 13032. *Microbiology* **157**: 77–88.
Jones, C.J., Utada, A., Davis, K.R., Thongsomboon, W., Zamorano Sanchez, D., Banakar, V., et al. (2015) C-di-GMP Regulates Motile to Sessile Transition by Modulating MshA Pili Biogenesis and Near-Surface Motility Behavior in *Vibrio cholerae*. *PLoS Pathog* **11**: e1005068.
Joshi, N. and Fass, J. (2011) Sickle: A sliding-window, adaptive, quality-based trimming tool for FastQ files (Version 1.33) [Software]. *Available at https://github.com/najoshi/sickle*.
Kelly, A.J., Sackett, M.J., Din, N., Quardokus, E., and Brun, Y. V (1998) Cell cycle-dependent transcriptional and proteolytic regulation of FtsZ in *Caulobacter*. *Genes Dev* **12**: 880–893.
Kharadi, R.R. and Sundin, G.W. (2019) Physiological and microscopic characterization of cyclic-di-GMP-mediated autoaggregation in *Erwinia amylovora*. *Front Microbiol* **10**:.
Laskos, L., Ryan, C.S., Fyfe, J.A.M., and Davies, J.K. (2004) The RpoH-mediated stress response in *Neisseria gonorrhoeae* is regulated at the level of activity. *J Bacteriol*.
Loto, F., Coyle, J.F., Padgett, K.A., Pagliai, F.A., Gardner, C.L., Lorca, G.L., and Gonzalez, C.F. (2017) Functional characterization of LotP from *Liberibacter asiaticus*. *Microb Biotechnol* **10**: 642–656.
De Lucena, D.K.C., Pühler, A., and Weidner, S. (2010) The role of sigma factor RpoH1 in the pH stress response of *Sinorhizobium meliloti*. *BMC Microbiol* **10**:.
McCutcheon, J.P. (2010) The bacterial essence of tiny symbiont genomes. *Curr Opin Microbiol* **13**: 73–78.
Moran, N.A. (2002) Microbial minimalism: Genome reduction in bacterial pathogens. *Cell* **108**: 583–586.
Motherway, M.O.C., Zomer, A., Leahy, S.C., Reunanen, J., Bottacini, F., Claesson, M.J., et al. (2011) Functional genome analysis of *Bifidobacterium breve* UCC2003 reveals type IVb tight adherence (Tad) pili as an essential and conserved host-colonization factor. *Proc Natl Acad Sci U S A*.
Naranjo, E., Merfa, M. V., Ferreira, V., Jain, M., Davis, M.J., Bahar, O., et al. (2019) *Liberibacter crescens* biofilm formation in vitro: establishment of a model system for pathogenic 'Candidatus Liberibacter spp.' *Sci Rep* **9**: 5150.
Nwugo, C.C., Duan, Y., and Lin, H. (2013) Study on citrus response to huanglongbing highlights a down-regulation of defense-related proteins in lemon plants upon "*Ca*. Liberibacter asiaticus" infection. *PLoS One* **8**: e67442.
O'Toole, G.A. (2010) Microtiter dish Biofilm formation assay. *J Vis Exp*.
Pagliai, F.A., Coyle, J.F., Kapoor, S., Gonzalez, C.F., and Lorca, G.L. (2017) LdtR is a master regulator of gene expression in *Liberibacter asiaticus*. *Microb Biotechnol* **10**: 896–909.
Pagliai, F.A., Gardner, C.L., Bojilova, L., Sarnegrim, A., Tamayo, C., Potts, A.H., et al. (2014) The Transcriptional Activator LdtR from "*Candidatus* Liberibacter asiaticus" Mediates Osmotic Stress Tolerance. *PLoS Pathog* **10**:.
Palmer, J., Flint, S., and Brooks, J. (2007) Bacterial cell attachment, the beginning of a biofilm. *J Ind Microbiol Biotechnol* **34**: 577–588.
Pan, L., Gardner, C.L., Pagliai, F.A., Gonzalez, C.F., and Lorca, G.L. (2017) Identification of the tolfenamic acid binding pocket in PrbP from *Liberibacter asiaticus*. *Front Microbiol* **8**:.
Pan, L., da Silva, D., Pagliai, F.A., Harrison, N.A., Gonzalez, C.F., Lorca, G.L., et al. (2019) The ferredoxin-like protein FerR regulates PrbP Activity in *Liberibacter asiaticus*. *Appl Environ Microbiol* **85**: e02605-18.
Persat, A., Stone, H.A., and Gitai, Z. (2014) The curved shape of *Caulobacter crescentus*

enhances surface colonization in flow. *Nat Commun* **5**: 3824.
Pitino, M., Armstrong, C.M., Cano, L.M., and Duan, Y. (2016) Transient expression of *Candidatus* Liberibacter asiaticus effector induces cell death in *Nicotiana benthamiana*. *Front Plant Sci* **7**: 982.
Prasad, S., Xu, J., Zhang, Y., and Wang, N. (2016) SEC-Translocon Dependent Extracytoplasmic Proteins of *Candidatus* Liberibacter asiaticus. *Front Microbiol* **7**: 1989.
Quon, K.C., Marczynski, G.T., and Shapiro, L. (1996) Cell cycle control by an essential bacterial two-component signal transduction protein. *Cell* **84**: 83–93.
Rammohan, J., Ruiz Manzano, A., Garner, A.L., Prusa, J., Stallings, C.L., and Galburt, E.A. (2016) Cooperative stabilization of *Mycobacterium tuberculosis* rrnAP3 promoter open complexes by RbpA and CarD. *Nucleic Acids Res* **44**: 7304–7313.
Rammohan, J., Ruiz Manzano, A., Garner, A.L., Stallings, C.L., and Galburt, E.A. (2015) CarD stabilizes mycobacterial open complexes via a two-tiered kinetic mechanism. *Nucleic Acids Res* **43**: 3272–3285.
Reisenauer, A., Quon, K., and Shapiro, L. (1999) The CtrA response regulator mediates temporal control of gene expression during the *Caulobacter* cell cycle. *J Bacteriol* **181**: 2430–9.
Rice, K.C., Mann, E.E., Endres, J.L., Weiss, E.C., Cassat, J.E., Smeltzer, M.S., and Bayles, K.W. (2007) The cidA murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* **104**: 8113–8118.
Sambrook, J., Fritsch, E.F., and Maniatis, T. (2001) Molecular Cloning: A Laboratory Manual. *Cold Spring Harb Lab* **3**: 2344.
Sangermani, M., Hug, I., Sauter, N., Pfohl, T., and Jenal, U. (2019) Tad pili play a dynamic role in.
Simm, R., Morr, M., Kader, A., Nimtz, M., and Römling, U. (2004) GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessibility to motility. *Mol Microbiol*.
Sisti, F., Ha, D.-G., O'Toole, G.A., Hozbor, D., and Fernández, J. (2013) Cyclic-di-GMP signalling regulates motility and biofilm formation in *Bordetella bronchiseptica*. *Microbiology* **159**: 869–879.
Skerker, J.M. and Shapiro, L. (2000) Identification and cell cycle control of a novel pilus system in *Caulobacter crescentus*. *EMBO J* **19**: 3223–34.
Sourjik, V., Muschler, P., Scharf, B., and Schmitt, R. (2000) VisN and VisR are global regulators of chemotaxis, flagellar, and motility genes in *Sinorhizobium (Rhizobium) meliloti*. *J Bacteriol* **182**: 782–788.
Spencer, W., Siam, R., Ouimet, M.-C., Bastedo, D.P., and Marczynski, G.T. (2009) CtrA, a global response regulator, uses a distinct second category of weak DNA binding sites for cell cycle transcription control in *Caulobacter crescentus*. *J Bacteriol* **191**: 5458–70.
Stallings, C.L., Stephanou, N.C., Chu, L., Hochschild, A., Nickels, B.E., and Glickman, M.S. (2009) CarD is an essential regulator of rRNA transcription required for *Mycobacterium tuberculosis* persistence. *Cell* **138**: 146–59.
Takenaka, T., Ito, T., Miyahara, I., Hemmi, H., and Yoshimura, T. (2015) A new member of MocR/GabR-type PLP-binding regulator of d -alanyl- d -alanine ligase in *Brevibacillus brevis*. *FEBS J* **282**: 4201–4217.
Taw, M.N., Lee, H.I., Lee, S.H., and Chang, W.S. (2015) Characterization of MocR, a GntR-like transcriptional regulator, in *Bradyrhizobium japonicum*: its impact on motility, biofilm formation, and soybean nodulation. *J Microbiol* **53**: 518–525.

Teixeira, D. do C. (2005) "*Candidatus* Liberibacter americanus", associated with citrus huanglongbing (greening disease) in Sao Paulo State, Brazil. *Int J Syst Evol Microbiol* **55**: 1857–1862.

Tomich, M., Fine, D.H., and Figurski, D.H. (2006) The TadV protein of *Actinobacillus actinomycetemcomitans* is a novel aspartic acid prepilin peptidase required for maturation of the Flp1 pilin and TadE and TadF pseudopilins. *J Bacteriol*.

Tomich, M., Planet, P.J., and Figurski, D.H. (2007) The tad locus: postcards from the widespread colonization island. *Nat Rev Microbiol* **5**: 363–375.

Tramonti, A., Nardella, C., Salvo, M.L., Pascarella, S., and Contestabile, R. (2018) The MocR-like transcription factors: pyridoxal 5′- phosphate- dependent regulators of bacterial metabolism. *FEBS J* **285**: 3925–3944.

Valentini, M. and Filloux, A. (2016) Biofilms and Cyclic di-GMP (c-di-GMP) signaling: Lessons from *Pseudomonas aeruginosa* and other bacteria. *J Biol Chem* **291**: 12547–12555.

Wairuri, C.K., Van Der Waals, J.E., Van Schalkwyk, A., and Theron, J. (2012) *Ralstonia solanacearum* needs Flp pili for virulence on potato. *Mol Plant-Microbe Interact*.

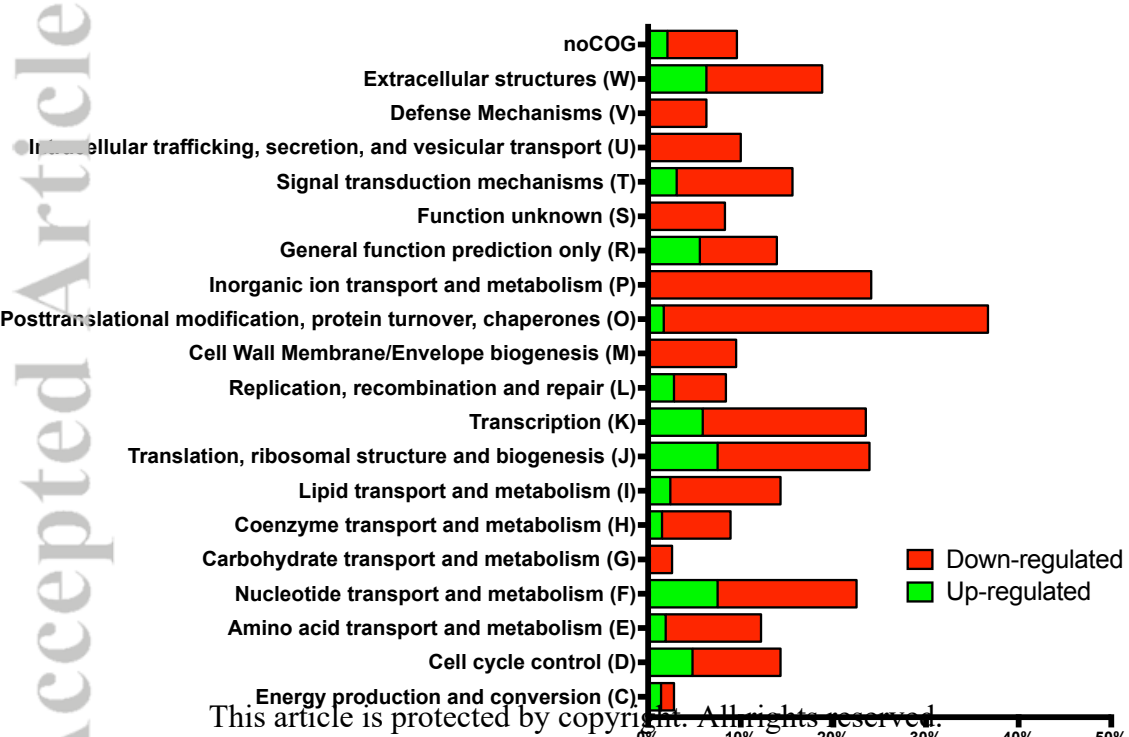
Wang, N., Pierson, E.A., Setubal, J.C., Xu, J., Levy, J.G., Zhang, Y., et al. (2017) The *Candidatus* Liberibacter-Host Interface: Insights into Pathogenesis Mechanisms and Disease Control. *Annu Rev Phytopathol* **55**: 451–482.

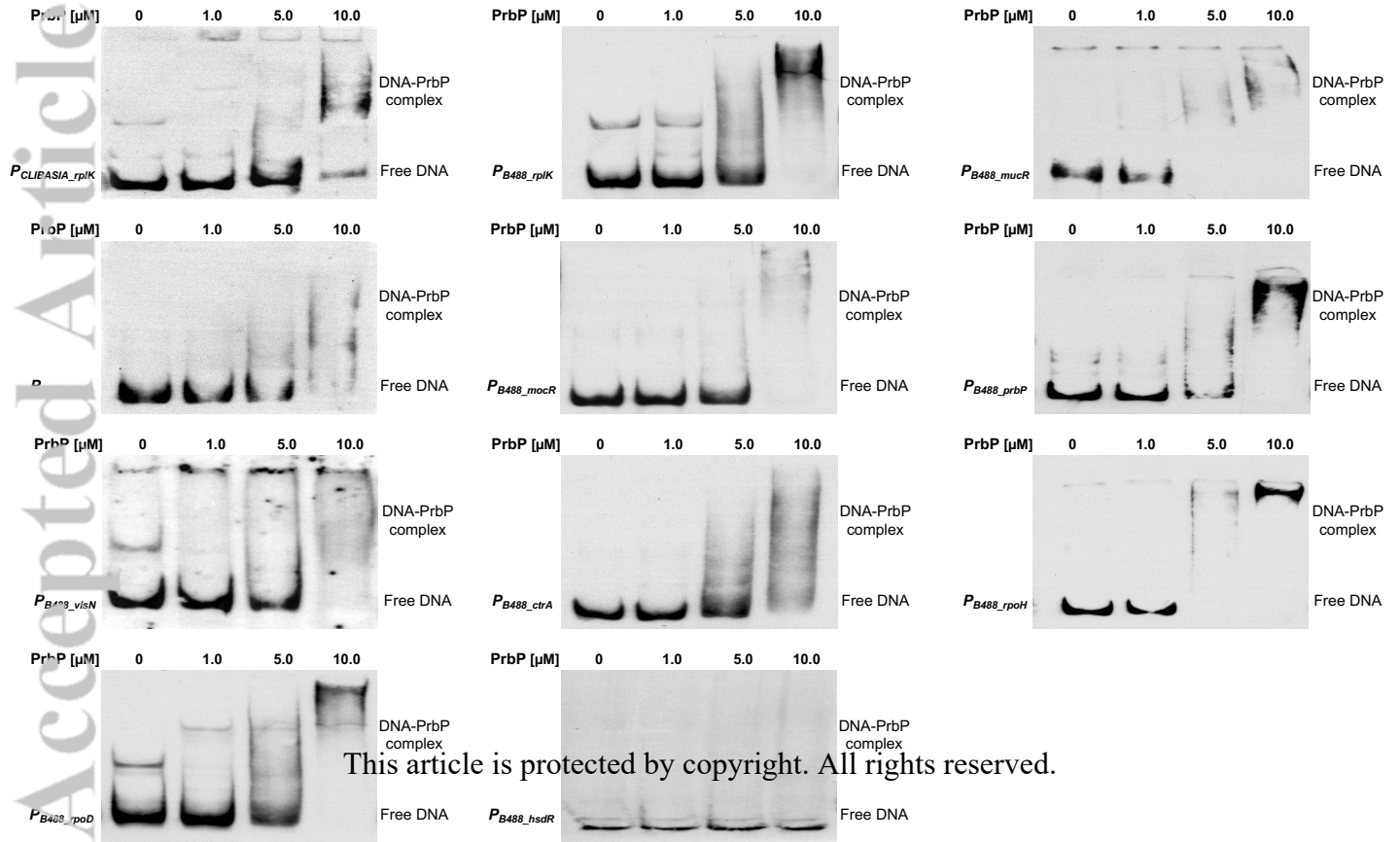
Weiss, L.A., Harrison, P.G., Nickels, B.E., Glickman, M.S., Campbell, E.A., Darst, S.A., and Stallings, C.L. (2012) Interaction of CarD with RNA Polymerase Mediates *Mycobacterium tuberculosis* Viability, Rifampin Resistance, and Pathogenesis. *J Bacteriol* **194**: 5621–5631.

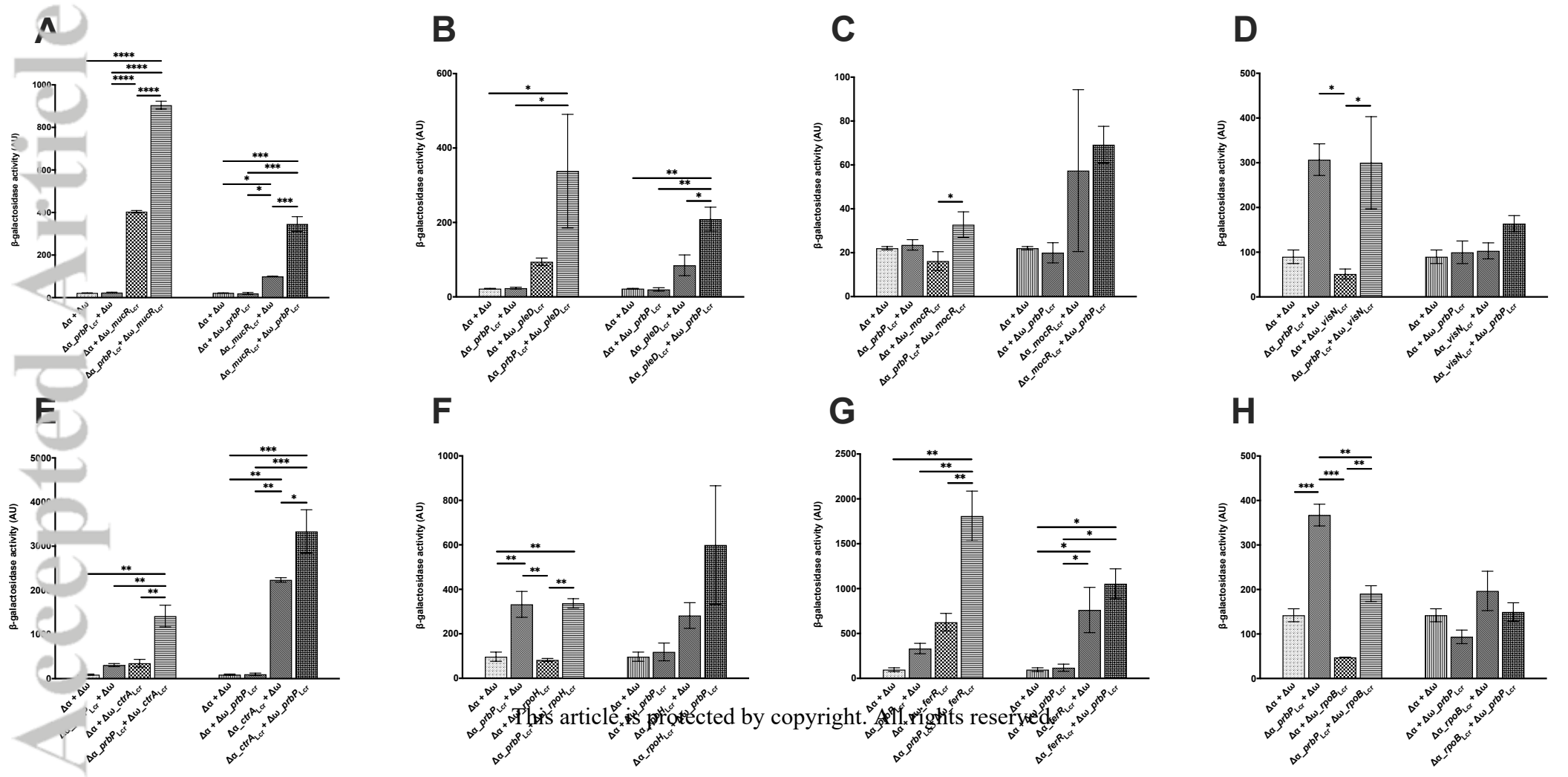
Yan, Q., Sreedharan, A., Wei, S., Wang, J., Pelz-Stelinski, K., Folimonova, S., and Wang, N. (2013) Global gene expression changes in *Candidatus* Liberibacter asiaticus during the transmission in distinct hosts between plant and insect. *Mol Plant Pathol* **14**: 391–404.

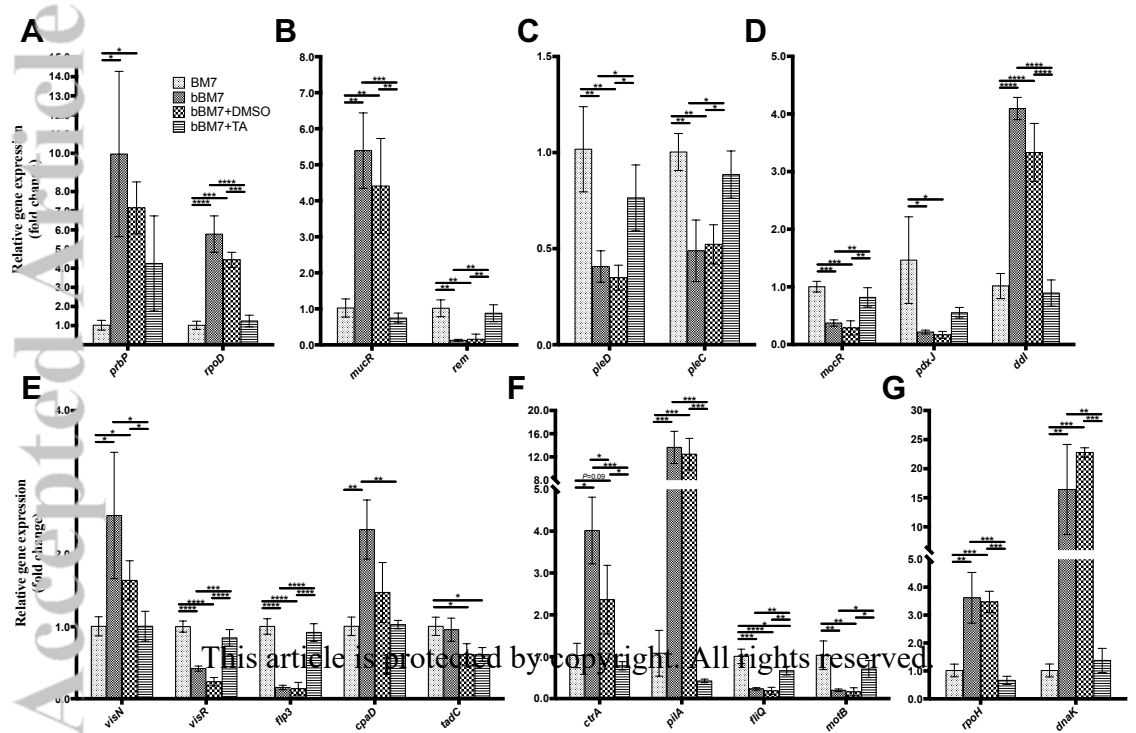
Zheng, Z.L. and Zhao, Y. (2013) Transcriptome comparison and gene coexpression network analysis provide a systems view of citrus response to "*Candidatus* Liberibacter asiaticus" infection. *BMC Genomics* **14**: 27.

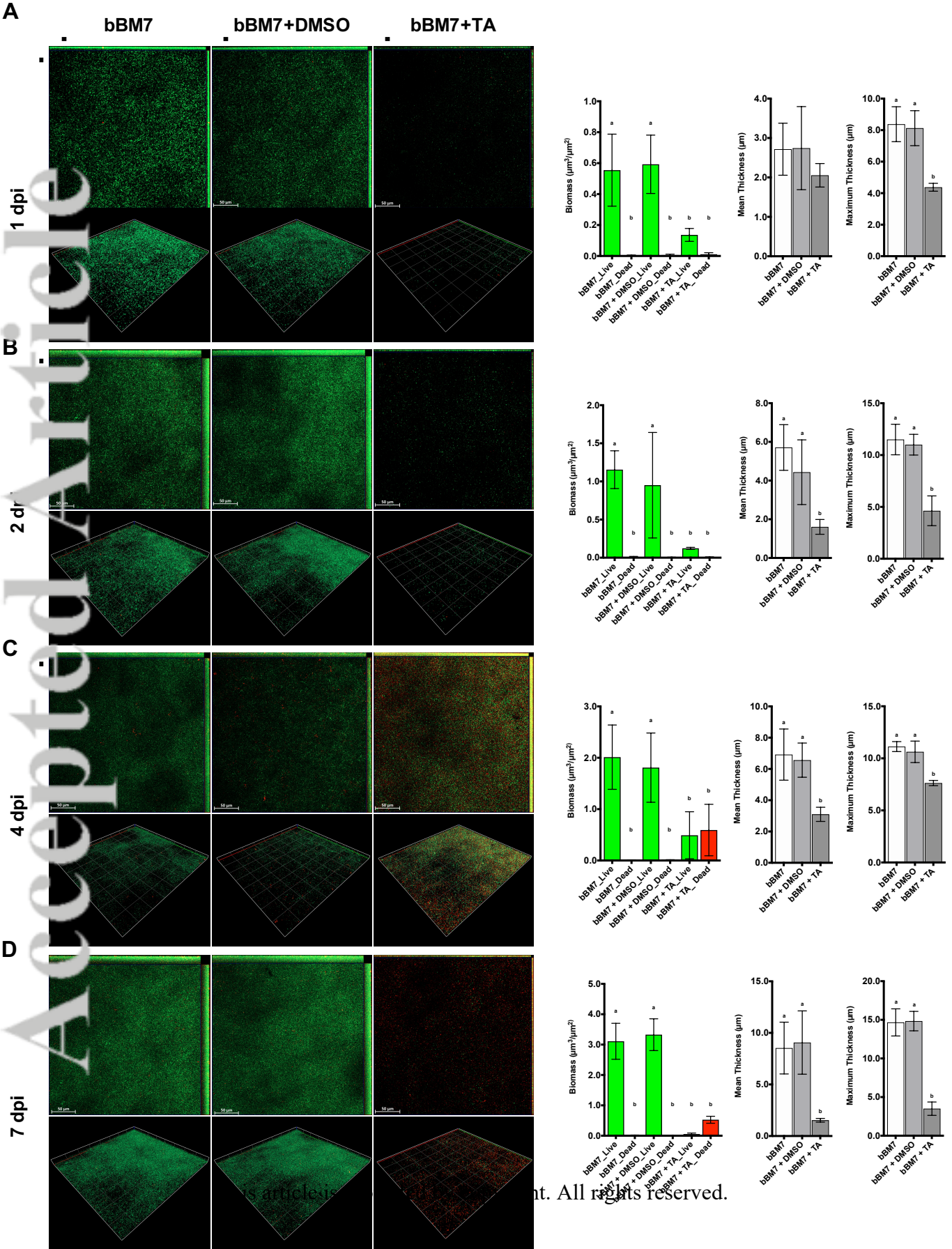
Zhu, D.X., Garner, A.L., Galburt, E.A., and Stallings, C.L. (2019) CarD contributes to diverse gene expression outcomes throughout the genome of *Mycobacterium tuberculosis*. *Proc Natl Acad Sci U S A* **116**: 13573–13581.











Chemotaxis; Motility

Pilus biogenesis; Biofilm formation

C-di-GMP

CtrA

VisR

VisN

PleD

PleC

Rem

MucR

PrbP

FerR

Exopolysaccharide biosynthesis

MocR

RpoD

RpoH

PLP synthesis; Peptidoglycan synthesis; Amino acid metabolism

Housekeeping genes

Heat shock response; General stress response

Transcription

Protein interaction

Repression

Activation