A high-throughput system to identify inhibitors of *Candidatus* Liberibacter asiaticus transcription regulators

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Citrus greening disease, also known as huanglongbing (HLB), is the most devastating disease of *Citrus* worldwide. This incurable disease is caused primarily by the bacterium *Candidatus* Liberibacter asiaticus and spread by feeding of the Asian Citrus Psyllid, *Diaphorina citri*. *Ca*. L. asiaticus cannot be cultured; its growth is restricted to citrus phloem and the psyllid insect. Management of infected trees includes use of broad-spectrum antibiotics, which have disadvantages. Recent work has sought to identify small molecules that inhibit *Ca*. L. asiaticus transcription regulators, based on a premise that at least some regulators control expression of genes necessary for virulence. We describe a synthetic, high-throughput screening system to identify compounds that inhibit activity of *Ca*. L. asiaticus transcription activators LdtR, RpoH, and VisNR. Our system uses the closely related model bacterium, *Sinorhizobium meliloti*, as a heterologous host for expression of a *Ca*. L. asiaticus transcription activator, the activity of which is detected through expression of an enhanced green fluorescent protein (EGFP) gene fused to a target promoter. We used this system to screen more than 120,000 compounds for compounds that inhibited regulator activity, but not growth. Our screen identified several dozen compounds that inhibit regulator activity in our assay. This work shows that, in addition to providing a means of characterizing *Ca*. L. asiaticus regulators, an *S. meliloti* host can be used for preliminary identification of candidate inhibitory molecules.

Liberibacter | *Sinorhizobium* | citrus | Huanglongbing (HLB) | transcription regulation

Citrus greening disease, also called huanglongbing (HLB), is catastrophic for world citrus industries (1, 2). The infecting agents are 3 bacterial *Candidatus* Liberibacter species, particularly *Candidatus* Liberibacter asiaticus (*C*Las) (3). *C*Las is spread between trees by the Asian citrus psyllid (ACP) (*Diaphorina citri*), a phloem-feeding insect that inoculates *Citrus* plants with bacteria from its salivary glands as it feeds on leaves (4).

*C*Las appears to cause disease by disrupting function of phloem, the essential vascular tissue that transports sugars and other nutrients from leaves (3). Early symptoms of HLB include yellowing of leaves (2), followed by dieback of both the canopy and fibrous roots (1). The few fruits that develop are misshapen, green, and bitter (2). There is no cure for HLB, and infection is terminal for the host tree. In the United States, active HLB disease was first discovered in Florida in 2005, where economic losses thus far have exceeded $4.5 billion. It has since spread to 2 other major citrus-growing states, Texas and California (1).

Since there is no effective treatment for infected trees, nor resistant commercial citrus varieties, HLB is managed mainly by controlling spread of the ACP vector and by replacing infected trees with uninfected nursery stock (1). Other measures that may ease HLB damage to citrus include maintaining optimal growth conditions, stimulating plant growth and defenses, thermotherapy of infected trees, biological control, and treatment with antimicrobials (1, 5). Regarding antimicrobials, streptomycin and oxytetracycline are permitted for foliar application in Florida under an emergency exemption (references cited in ref. 6), and there is much interest in identifying additional compounds that inhibit *C*Las infection and growth (1, 6, 7).

*C*Las is a reduced-genome, α-proteobacterium (8, 9) that cannot be cultured, precluding use of direct screens for antimicrobial discovery. The only known commensal *Liberibacter*, *Liberibacter crescens*, can be cultured and is being developed as a model system to study *Liberibacter* physiology and genetics, including response to antimicrobial treatments, but still lacks the tools of better studied α-proteobacteria (10–18). *C*Las is closely related to the beneficial nitrogen-fixing plant symbiont *Sinorhizobium meliloti* (*Sme*), which has been used as a heterologous host to express specific *C*Las genes (14). Construction of a flexible synthetic-model system using highly tractable *Sme* could allow in vivo screening for discovery of new treatments.

The vast majority of commercial antimicrobials target essential bacterial functions. However, these antimicrobials present a significant downside: By targeting essential cellular processes, they exert selective pressure that allows resistant bacterial populations to emerge. The rise of drug-resistant pathogens has resulted in increased interest in narrow-spectrum and targeted approaches against microbial pathogens, such as those focusing on specific signaling and virulence pathways (19, 20).

One approach in targeting virulence pathways is to identify inhibitors of the actual proteins that are directly responsible for the disease symptoms (19). This is not feasible for many pathogens

Significance

The insect-disseminated bacterium *Candidatus* Liberibacter asiaticus causes the destructive, incurable "citrus greening disease," which is widespread in Asia, Africa, and the Americas, resulting in economic losses in the billions of dollars. One approach to treating infected citrus trees is application of antimicrobial compounds. However, use of broad-spectrum antibiotics in commercial citrus orchards has significant disadvantages, such as emergence of resistance and inhibition of beneficial bacteria. We designed a synthetic system for high-throughput screening of compound libraries in a closely related, culturable, and nonpathogenic bacterium, allowing us to identify small molecule inhibitors of *Ca*. L. asiaticus transcription activators.

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Data deposition: The sequences reported in this paper have been deposited in GenBank, https://www.ncbi.nlm.nih.gov/genbank (accession nos. MK359043–MK359048 and MK387175). Affymetrix GeneChip data have been deposited in the Gene Expression Omnibus (GEO) database, https://www.ncbi.nlm.nih.gov/geo (Superseries accession no. GSE124984).

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because pathogenesis mechanisms are poorly defined: This is the case with *C*Las. Inhibiting regulatory proteins such as transcription factors is an alternative strategy that proved successful in other bacteria (20–25). The small number of predicted regulators encoded by *C*Las (8, 9) makes it feasible to systematically screen for small molecule inhibitors in a high-throughput manner. We therefore designed an in vivo synthetic system to screen for inhibitors of *C*Las transcription activators, using the closely related model bacterium, *S. meliloti*, as a heterologous host. Our approach identified candidate compounds that inhibited activity of *C*Las transcription activators and complements recent in vitro inhibitor screens (11, 14, 26, 27).

Results

CLas Encodes up to 19 Transcription Regulators. Our bioinformatic analyses predict that the *C*Las genome encodes 19 transcription regulators, including 2 sigma factors: RpoD (housekeeping sigma factor) and RpoH (likely heat shock/stress response sigma factor) (Table 1 and *SI Appendix*, Table S1). All but one appear to have orthologs in the related, beneficial nitrogen fixing symbiont, *Sme*. Work in *Sme* and *L. crescens* (13) suggests that 6 *C*Las regulators are essential for viability: RpoD, RpoH, CtrA, DivK, GcrA, and CpdR1. The last 4 of these may play critical roles in *C*Las cell cycle regulation (28). Some of the 19 predicted *C*Las regulators may not modulate gene expression: The sole *C*Las LexA-like regulator (GenBank accession no. ACT56917) appears to lack a helix-turn-helix DNA binding domain; *C*Las lacks a σ^{54}-type sigma factor to act in concert with its putative TacA-like enhancer-binding protein (ACT57389).

In deciding which *C*Las regulators to study, we considered 3 main factors: characteristics of the *Sme* ortholog(s); representation of multiple regulator families, since the 12 different regulator types present in *C*Las are expected to be inhibited differently; and *C*Las expression pattern, because regulators involved in citrus virulence may show increased in planta gene expression. For expression pattern, we consulted an RT-qPCR study that compared expression of 381 *C*Las genes amplified from infected sweet orange host plants versus *C*Las-harboring psyllid insects (29). Based on these factors, we chose the following 6 regulators, all predicted to function as transcription activators, for transcriptome analysis and high-throughput inhibitor screening: RpoH, VisNR, LdtR, LsrB, PhrR, and CtrA (Table 1).

CLas Regulators Can Be Expressed Efficiently in *S. meliloti*. Our high-throughput screening approach for identifying inhibitory compounds required that the *C*Las regulator was expressed well in its heterologous *Sme* host. We optimized for plasmid copy number, exogenous promoter used for expression, ribosome binding site (RBS), and codon usage (*SI Appendix*, *Materials and Methods*). To decrease background transcription levels of target genes whose expression may be activated by both the *Sme* and the *C*Las regulator, we introduced regulator expression plasmids into strains deleted for the *Sme* orthologous regulator(s), except for *ctrA* (*SI Appendix*, Table S2).

We performed phenotypic assays to determine if the *C*Las regulator could compensate for defects caused by deletion of the *Sme* orthologous regulators (*SI Appendix*, *Materials and Methods*). We observed qualitative growth of all of the strains, and in certain strains we also assessed heat stress, swimming motility, cell morphology, and cell envelope integrity. To identify genes whose expression increased with ectopic expression of the *C*Las regulator, we performed Affymetrix GeneChip analysis on *Sme* strains deleted for the orthologous *Sme* regulator(s). These carried either a plasmid that encoded the *C*Las regulator or the empty vector and were induced with Isopropyl β-D-1-thiogalactopyranoside (IPTG) to provide strong expression of the heterologous regulator. Phenotypic and transcriptome results for each of these 6 strains are detailed in the sections below.

RpoH. *Sme* RpoH1 and RpoH2 alternative sigma factors mediate response to various stressors, including heat, acid, hydrogen peroxide, stationary phase growth, and envelope disrupting agents (30–33). *C*Las RpoH is most similar to *Sme* RpoH1 (72% identity), which is required for an effective nitrogen-fixing symbiosis (32, 34). *C*Las RpoH and *Sme* RpoH1 both complemented a *Sme* Δ*rpoH1rpoH2* strain for growth at restrictive temperature.

To identify transcripts whose abundance increased in *Sme* Δ*rpoH1rpoH2* when *C*Las RpoH was ectopically expressed, we performed Affymetrix GeneChip analysis, with the same strain expressing *Sme rpoH1* as a positive control, and the empty vector

Table 1. CLas transcription regulators chosen for study

CLas regulator, GenBank accession no.*	*S. meliloti* (*Sme*) ortholog(s)†	Percent identity between CLas and *Sme* proteins	Regulator type	Putative function of regulator in *Sme* (ref.)	CLas fold change expression for plant vs. psyllid‡
ACT57084	RpoH1, RpoH2	72, 41	Sigma factor	Stress response, symbiosis (30–34)	2.6
ACT57167	VisN	50	LuxR	Motility; forms heterodimer with VisR (35)	6.7
ACT57166	VisR	49	LuxR	Motility; forms heterodimer with VisN (35)	4.3
ACT56824	LdtR (SMc01768)	70	MarR	Osmotic stress tolerance, peptidoglycan remodeling (14)	37.1
ACT56755	LsrB (SMc01225)	58	LysR	LPS biosynthesis, symbiosis (42, 54)	4.7
ACT56897	PhrR1 (SMc01110), PhrR2 (SMb21117)	59, 48	HTH-XRE	Quorum sensing (43)	Not reported
ACT57366	CtrA	75	Response regulator	Cell cycle control; essential gene (40)	3.3

*Accession numbers are for regulators in the CLas Psy62 genome, assembled from a psyllid metagenome (8). *SI Appendix*, Table S1 lists other putative CLas transcription regulators not chosen for this study.

†If the gene name has not been annotated in GenBank, the *S. meliloti* 1021 unique locus tag is given in parentheses.

‡Data published in Yan et al. (29). The fold change provided here was calculated from their reported $\log_2$ ratio values. Not reported means the gene was listed in Yan et al's. supplementary table 1 as "selected for qRT-PCR analysis," but a $\log_2$ ratio was not reported.

(pSRKGm) as a point of reference. Eight genes showed increased expression ≥2-fold with both *C*Las RpoH and *Sme* RpoH (Dataset S1): All were previously identified as RpoH1-dependent in other studies (30, 31).

VisNR. VisN and VisR function as a heterodimer to positively regulate chemotaxis, flagellar, and motility genes (35) in *Sme* and negatively regulate the *flp3* pilus gene in *C*Las (36). The *C*Las genome contains genes predicted for flagellar biogenesis and motility (8), but it is unknown if *C*Las forms functional flagella. The *Sme* Δ*visNR* deletion strain was nonmotile on soft agar plates, and expression of *C*Las *visNR* restored WT motility. Transcriptomic comparisons of *Sme* Δ*visNR* expressing *C*Las *visNR* versus the empty vector revealed that, as expected, *C*Las *visNR* stimulates expression of *Sme* motility and chemotaxis genes (Dataset S1). The most strongly expressed gene was *rem* (11-fold), which encodes a response regulator that acts downstream of *Sme* VisNR to activate motility gene expression during exponential growth (37).

LdtR. *Sme* LdtR was postulated to play a role in response to hyperosmotic stress, perhaps by activating expression of the adjacent gene, *ldtP*, which encodes a putative L,D-transpeptidase (14, 38). Work in *L. crescens* implies that LdtR is a master regulator controlling diverse functions, including motility and cell wall biogenesis (39). *Sme* Δ*ldtR* had a swimming motility defect (~30% of WT motility), which was not suppressed by ectopically expressing *C*Las LdtR. Expressing *C*Las LdtR in WT *Sme* also reduced motility, implying that both too much and too little *Sme* LdtR is deleterious for motility. Ectopically expressing either *C*Las or *Sme* LdtR in either WT or Δ*ldtR* strains resulted in poor growth on Luria Broth (LB) medium; because of this, and because previous work indicated a role for LdtR in cell wall remodeling (14, 38, 39), we examined cellular morphology of WT strains expressing either *C*Las or *Sme* LdtR (*Materials and Methods*). Compared to WT carrying the empty vector, cells expressing either *C*Las or *Sme* LdtR were elongated, branched, and had bulges (*SI Appendix*, Fig. S1). We compared the transcriptome of *Sme* Δ*ldtR* expressing *C*Las LdtR versus the empty vector strain; only 8 genes showed ≥1.5-fold increase in expression (Dataset S1). Five of these may be controlled by the master cell cycle regulator, CtrA (40), including *tacA*, which had the highest increase in expression (~10-fold), and whose *Caulobacter crescentus* ortholog was shown to be a global cell cycle regulator involved in polar development (41).

LsrB. *Sme* LsrB activates expression of oxidative stress-related genes and an operon involved in lipopolysaccharide biosynthesis (*lrp3-lpsCDE*) (42), which likely explains why the Δ*lsrB* mutant grew poorly and was >1,000-fold more sensitive than WT to the envelope-disrupting detergent, deoxycholate (DOC). Expression of *C*Las *lsrB* only partially suppressed the DOC-growth defect of Δ*lsrB*; this strain was ~10- to 100-fold more sensitive to DOC than was WT. Comparison of transcriptome profiles for *Sme* Δ*lsrB* expressing *C*Las LsrB compared to the control identified only 3 genes with expression increased ≥1.2-fold, while genes in the *lrp3-lpsCDE* operon failed to show increased expression (Dataset S1).

PhrR. *Sme* has 2 orthologs of *C*Las PhrR (Table 1). PhrR1 may play a role in quorum sensing (QS) because the ExpR QS regulator represses *phrR1* expression by binding upstream of *phrR1*, in an acyl homoserine lactone-dependent manner (43). In closely related *S. medicae*, *phrR1* expression increases in response to stresses such as low pH, ethanol, zinc, copper, and H_2O_2 (44). Both Δ*phrR1* and Δ*phrR1phrR2* mutants grew more slowly than WT on LB plates. During RNA purification, the Δ*phrR1phrR2* mutant was more resistant to lysozyme lysis, suggesting an alteration of its lipopolysaccharide or other envelope component (*SI Appendix*, *Materials and Methods*). We identified no genes whose expression increased ≥1.5-fold in *Sme* Δ*phrR1phrR2* expressing *C*Las PhrR (Dataset S1).

CtrA. CtrA is essential for viability in *Sme* and most other α-proteobacteria (40). When we expressed *Sme ctrA* from a plasmid, we could delete the genome copy of *Sme ctrA* but not when *C*Las *ctrA* was similarly expressed. Therefore, we used a WT *Sme* host to compare transcriptome profiles for cells expressing *C*Las *ctrA* or *Sme ctrA* vs. empty vector. Genes whose expression increased with *C*Las *ctrA* (Dataset S1) included the *minCDE* operon, encoding proteins that inhibit septum formation at appropriate times during the cell cycle (40), and *ldtR*. This latter result is intriguing given that expressing *C*Las *ldtR* appears to decrease expression of CtrA-dependent genes (see above). *Sme* CtrA directly represses *minCDE* expression during the cell cycle (40); perhaps increased expression of *minCDE*, caused by ectopic expression of *C*Las *ctrA*, contributes to the growth defects observed in these strains.

High-Throughput Screening Identified Compounds That May Inhibit *C*Las Transcription Regulators. By identifying genes whose expression increased when IPTG induced expression of each of the 6 *C*Las regulators, we defined promoters targeted by each of them. Candidate promoters were fused to an enhanced green fluorescent protein (EGFP) reporter gene for high throughput compound screening. We designed the screening strains to have low basal fluorescence and to fluoresce strongly upon IPTG-induced expression of the *C*Las regulator. Our easy-to-use custom expression cassette has features to optimize signal over noise (*SI Appendix*, Fig. S2). After cloning this synthetic EGFP cassette into each of the 6 regulator gene-containing plasmids, we cloned each of 11 promoters (chosen as described in *Materials and Methods*) into the appropriate plasmid, then introduced each plasmid into the appropriate *Sme* deletion strain (*SI Appendix*, Table S2).

Based on qualitative fluorescence assays, we selected 3 regulator-promoter constructs for further testing at the Stanford High-Throughput Bioscience Center (HTBC): LdtR (P*smc04059*), RpoH (P*ibpA*), and VisNR (P*rem*). Strains with these constructs performed well in pilot experiments: fluorescence in IPTG-induced cells was high compared to the basal level (Fig. 1). High-throughput screening of 10 libraries (>120,000 compounds) was performed for each of these strains (*Materials and Methods* and *SI Appendix*, Fig. S3 and Table S3).

The Known Bioactive Collection was screened first to evaluate the performance of our screening assay; most of these libraries were screened at 7 different concentrations (*SI Appendix*, Fig. S3 and Table S3). Screening at multiple concentrations allowed us to plot % fluorescence and absorbance inhibition versus compound concentration, and the presence of known antibacterial compounds in these libraries ensured that we would detect at least some inhibitory compounds. Over 130 compounds inhibited growth by at least 50% in all 3 testing strains (Dataset S2). As expected, these inhibitory compounds, most of which have known antibacterial activity, also inhibited EGFP fluorescence, validating the assay. However, our goal was to identify compounds that decreased function of a *C*Las regulator without severely inhibiting growth to avoid compounds that could be broadly toxic in a natural environment and that would increase selective pressure for resistant bacteria. The desirable compounds should show high inhibition of EGFP fluorescence but low or no inhibition of growth as measured by absorbance. We arbitrarily set cutoffs for inhibition of EGFP fluorescence ≥30%, and <50% for growth inhibition, with a difference between EGFP fluorescence and growth inhibition of ≥30%. We also eliminated from consideration compounds that inhibited EGFP fluorescence of all 3 strains, because such compounds may generally inhibit GFP activity or quench fluorescence. Dataset S2 lists 69 compounds from the Bioactive Collection meeting the above criteria. The majority of these compounds specifically inhibited EGFP fluorescence in the LdtR strain (n = 45), while 13 compounds were specific for the VisNR strain and 4 for the RpoH strain.

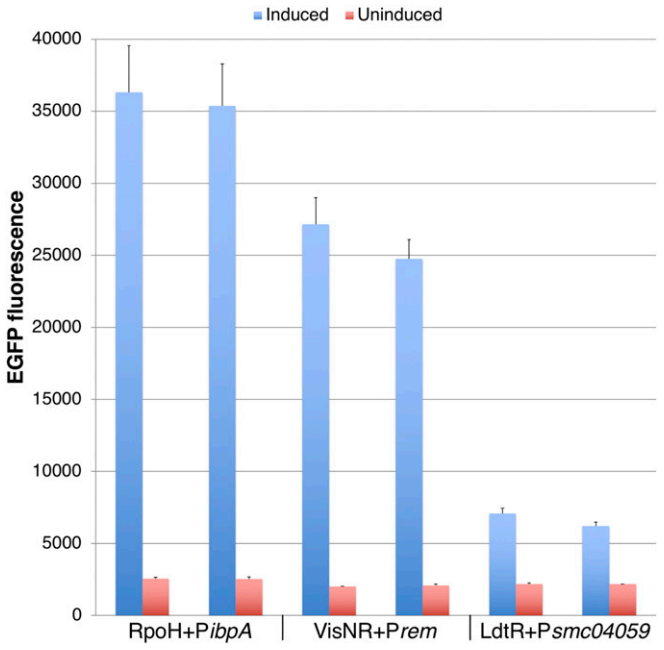

Fig. 1. EGFP fluorescence for IPTG-induced and uninduced high-throughput screening strains. Each *S. meliloti* strain was tested in duplicate 384-well plates for EGFP fluorescence. Each column indicates the average of raw signal values for IPTG-induced (n = 352) and uninduced (n = 16) wells. Error bars indicate SD. The *C*Las transcription regulator and *S. meliloti* promoter of each strain are indicated below the x axis. Mean EGFP fluorescence for M9 sucrose medium negative controls was 293 (n = 96). Strains: Δ*rpoH1rpoH2* *C*Las-RpoH P*ibpA* (MB231 pMB949); Δ*visNR* *C*Las-VisNR P*rem* (MB1102 pMB956); Δ*ldtR* *C*Las-LdtR P*smc04059* (MB1101 pMB958).

Seven compounds inhibited 2 strains. The small number of compounds inhibiting the RpoH strain may reflect a general lack of susceptibility of RpoH sigma factors to inhibition by small molecules; however, fluorescence and absorbance measurements were more variable for the RpoH plates than for the others. This variability could have led to a high proportion of false negative compounds.

Because the 113,809 compounds from the ChemDiv, ChemBridge, and Specs libraries in the HTBC's "Diverse Collection" were initially screened at a single concentration (*SI Appendix*, Fig. S3 and Table S3), we devised a ranking system for rescreening (*SI Appendix*, Tables S4 and S5) to ensure that efforts were evenly distributed among the regulators. The system prioritized compounds with greater differences between EGFP fluorescence and growth inhibition, and that specifically inhibited only 1 of the 3 regulators. In all, 629 compounds (0.55%) were rescreened in duplicate at 8 different concentrations. Rescreening identified 61 compounds that met the same criteria, described above for the Bioactive Collection, with strain specificities as follows: LdtR, 37; VisNR, 19; RpoH, 2; and inhibiting both RpoH and VisNR, 3 (Dataset S3). Although the candidate inhibitory compounds have diverse structures, we noticed regulator-specific patterns: 7 compounds inhibiting LdtR EGFP fluorescence possess 1,3-thiazole groups, and 9 compounds inhibiting VisNR EGFP possess sulfone groups.

To confirm the accuracy of the high-throughput screening results, we purchased 10 compounds (Fig. 2 and *SI Appendix*, Fig. S4) and retested them using a slightly different procedure than that of the original high-throughput screen. To gain information about specificity of inhibition, we also tested these 10 purchased compounds on each of the 3 *Sme* deletion strains carrying the corresponding *Sme* regulator (LdtR, RpoH1, VisNR) on a plasmid. Results are given in *SI Appendix*, Table S6, and for *C*Las regulators, the original screening results are provided for comparison.

Retesting results varied by compound and could be sorted into 3 groups by behavior (*SI Appendix*, Table S6). 1) One compound (ChemDiv 8013-5939) did not affect EGFP fluorescence or growth. 2) Four compounds affected growth (and therefore EGFP fluorescence) but showed no specificity for a single regulator (ChemBridge 5109513, Fisetin, Orbifloxacin, and Oxybenzone). 3) Results for 5 compounds mostly replicated our high-throughput screening data (4-Demethylepipodophyllotoxin, ChemDiv C549-0604, Bortezomib, ChemDiv D244-0326, and Rosiglitazone maleate). The latter 3 compounds inhibited EGFP fluorescence in VisNR and one or both RpoH strains. The overall pattern of results is consistent with inhibitors that affect expression, not EGFP function per se.

The most promising results were obtained with ChemDiv C549-0604, which consistently and strongly inhibited EGFP fluorescence in the *C*Las VisNR strain (IC_{50} = 0.7 μM; *SI Appendix*, Table S6). Since both *C*Las and *Sme* VisNR rescued a nonmotile *S. meliloti* Δ*visNR* strain, we tested whether ChemDiv C549-0604, D244-0326, and bortezomib decreased *C*Las and *Sme* VisNR-mediated motility. ChemDiv C549-0604 decreased motility of Δ*visNR* *C*Las pVisNR by 28%, but motility of Δ*visNR* *Sme* pVisNR was not affected (*SI Appendix*, Fig. S5). Neither ChemDiv D244-0326, nor bortezomib, affected motility. In summary, these results support the validity of our high-throughput screening methods, while highlighting the importance of multiple assays to retest candidate positive results.

Discussion

We carried out a high-throughput screen to identify compounds that inhibit activity of *C*Las transcription activators, without substantially inhibiting bacterial growth. Because *C*Las cannot be cultured, use of a closely related, genetically tractable, heterologous *Sme* host bacterium was the key feature of our in vivo screen design. Of 6 initial *C*Las regulators examined (CtrA, LdtR, LsrB, PhrR, RpoH, VisNR), 3 were chosen for high-throughput screening

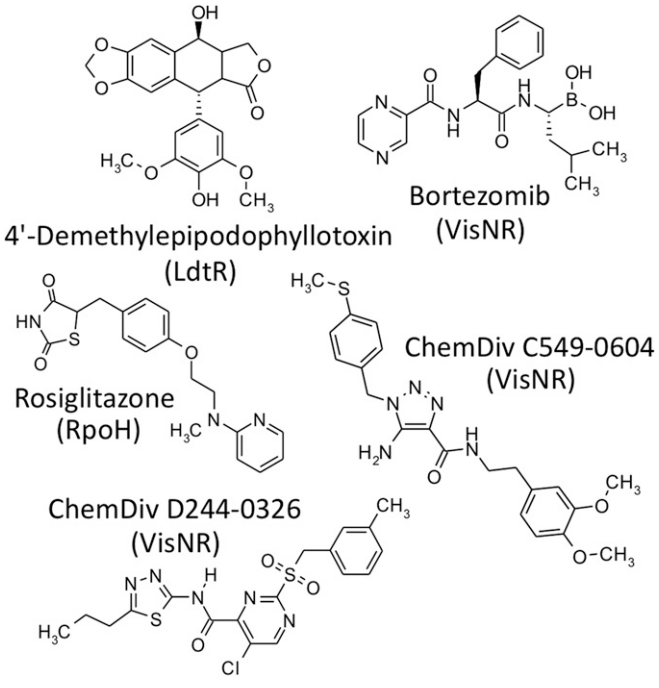

Fig. 2. Five inhibitory compounds whose effects on the *C*Las and *S. meliloti* regulators (LdtR, RpoH/RpoH1, and VisNR) were confirmed by purchasing compounds and retesting (*SI Appendix*, Table S6). Regulators identified via high-throughput screening as the putative targets of the inhibitory compounds are shown below the compound name (Bioactive Collection compounds) or supplier/catalog number (Diverse Collection compounds) in parentheses.

(LdtR, RpoH, VisNR), yielding candidate inhibitory (i.e., "lead") compounds. This work demonstrates the practicality of using the heterologous *Sme* host to study *C*Las regulator function. While *C*Las LdtR, RpoH, and VisNR seem to function similarly to their orthologous *Sme* proteins, *C*Las CtrA, LsrB, and PhrR may have distinct functions.

Both in vitro and in vivo assays have been used to screen for inhibitors of transcription regulators. In vitro approaches include using purified proteins to screen for compounds that differentially affect temperature-dependent protein unfolding (e.g., differential scanning fluorimetry [DSF]) (45) and cell-free DNA binding assays (21). DSF combined with DNA-binding assays successfully identified small molecule inhibitors of *C*Las regulators, LdtR and PrbP (11, 14, 26, 27). While in vitro approaches are valuable, there are downsides to their use: In vitro screening requires sufficient amounts of purified, active target protein. In the case of DSF, it is unclear whether the degree of thermal denaturation is an accurate surrogate for in vivo protein inhibition. In vivo whole-cell, high-throughput approaches have succeeded in targeting transcription regulators (24, 25, 46–49), but these typically require laboratory culture of the bacterial species and at least partial replication of its natural environment.

We compared our results from screening the *C*Las LdtR *Sme* strain to data from the *C*Las LdtR DSF study (14). Our screen included 6 compounds that Pagliai et al. identified as affecting LdtR temperature-dependent protein unfolding, DNA binding, and/or *Sme* growth (Benzbromarone, Diethylstilbestrol, Hexestrol, Oxantel pamoate, Phloretin, Resveratrol). Of these 6 compounds, hexestrol inhibited both EGFP fluorescence and growth of all 3 *S. meliloti* strains (IC_{50} values ranging from 7 to 20 μM). Diethylstilbestrol inhibited EGFP fluorescence of all 3 strains in just 1 of 2 different libraries we screened (IC_{50} values = 13–18 μM). The remaining 4 compounds reported by Pagliai et al. were inactive in our screen, perhaps because we screened at lower concentrations (*SI Appendix*, Table S3) than were previously found to affect DNA binding and growth (50–250 μM) (14). The compound that most specifically inhibited EGFP fluorescence of our *C*Las LdtR strain (4-Demethylepipodophyllotoxin) was not among the 1,312 compounds screened previously (14). A thorough comparison of in vitro vs. in vivo screening methods will require studies of additional regulators, but this preliminary comparison suggests the 2 methods are complementary.

While use of a heterologous host has an obvious advantage in probing transcription of an unculturable bacterial species, our study confirms some downsides to this approach. For example, if *C*Las regulator function is distinct from that of its *Sme* ortholog, then transcriptome analysis with Affymetrix *Sme* GeneChips may fail to identify *Sme* target promoters sufficiently activated for use in a high-throughput screen that relies on EGFP fluorescence, as was the case for CtrA, LsrB, and PhrR. For such nonhomologous regulators, one could forego transcriptome analysis and instead screen a library of short, random *C*Las DNAs cloned into the regulator-EGFP vectors and look for robust EGFP fluorescence. Promoters that are activated only when the regulator is induced could then be further assessed for suitability in a high-throughput screen. Another option would be use of a more closely related host such and *L. crescens*, which is being developed as a model system, although *L. crescens* grows more slowly than *Sme* and has complex nutritional requirements (10–17).

Another downside of our screen design is that minimizing contribution of native *Sme* regulators to target promoter activity fluorescence necessitated deletion of the orthologous *Sme* regulator(s). If one of these regulators is critical for *Sme* growth/viability and the *C*Las regulator cannot complement its function, then the bacteria may have growth or other defects that complicate high-throughput screening. For example, *Sme* Δ*phrR* exhibited slow growth that was not suppressed by expressing *C*Las PhrR. A more severe example is that of *C*Las CtrA, whose expression not only failed to rescue viability of a *Sme* Δ*ctrA* strain, but was itself deleterious when expressed in WT *Sme* on LB growth medium. Although these issues precluded efforts using our design to screen for inhibitors of *C*Las CtrA, they suggest that simply screening for growth of WT *Sme* expressing *C*Las CtrA could identify compounds that inhibit activity of *C*Las CtrA, but not *Sme* CtrA.

Overall, our work demonstrates that it is feasible to identify *C*Las lead compounds using an in vivo fluorescence-based screen in a *Sme* host. This screen identified dozens of potential inhibitors of *C*Las transcription activators. Many of the "Known Bioactive" library compounds that inhibit EGFP fluorescence are unsuitable for treatment of citrus greening disease because of their toxicity, mutagenicity, or cost; however, results obtained with these compounds suggest that our screening system could be extended to assay nontoxic chemical fragments and related compounds for efficacy in combatting citrus greening. Screening the "Diverse Collection" library identified lead compounds, the most promising of which (ChemDiv C549-0604) appears to specifically inhibit activity of *C*Las VisNR.

Research on the *C*Las-psyllid-Citrus disease triad is hampered by many technical challenges, such as inability to culture the *C*Las bacterial pathogen, variable insect behavior and ecology, and a large, perennial host plant that takes years to mature (50, 51). Because a single breakthrough discovery is unlikely to win the fight against citrus greening disease, progress depends on continued broad efforts by researchers across many disciplines. Microbiologists have focused on: understanding how *C*Las is transmitted and survives in its hosts; characterizing multiple *C*Las genomes; identifying *C*Las virulence functions and secreted effector proteins; discerning barriers to *C*Las laboratory cultivation; developing the culturable *L. crescens* model system; defining *Citrus* and psyllid microbiomes; and seeking means to control *C*Las with antagonistic bacteria, bacteriophages, and antimicrobials (50). The ultimate goal in the war on citrus greening is *C*Las-resistant commercial *Citrus* varieties, a difficult and likely long-range goal for numerous reasons, including lack of knowledge on how *C*Las interacts with *Citrus* at a molecular level. As long as barriers to *C*Las laboratory cultivation exist, such molecular mechanisms will be extremely difficult to dissect; thus, research using model heterologous systems such as *L. crescens*, *S. meliloti*, and other related α-proteobacteria will continue to be an important stopgap measure. Researchers studying *C*Las should continue to exploit the extensive knowledge available for α-proteobacteria, and we encourage researchers studying model α-proteobacteria to participate in combatting this modern agricultural plague.

Materials and Methods

Additional information is provided in *SI Appendix, Materials and Methods*.

Strains and Plasmids. Standard techniques were used for cloning, PCR amplification, strain constructions, and phenotypic assays (*SI Appendix, Materials and Methods*). All *Sme* strains used in this study are derived from CL150 (*SI Appendix*, Table S2). Oligonucleotide primers used in this study are listed in *SI Appendix*, Table S7. Regulator genes (Table 1) were cloned into the medium copy, IPTG-inducible vector, pSRKGm (52). We fully optimized the coding sequence of each *C*Las Psy62 (8) regulator for expression in *Sme* (GenBank accession nos.: MK359043–MK359048). Unmarked deletions of *Sme ctrA*, *ldtR*, *lsrB*, *phrR1*, *phrR2*, and the *visN-visR* operon were constructed.

Affymetrix GeneChip Analysis. For transcriptome analysis of *Sme*, we used a custom dual genome Affymetrix Symbiosis Chip (53). Each optimized *C*Las regulator was ectopically expressed by inducing with 0.5 mM IPTG. Controls for comparison were *Sme* deletion strains carrying the empty pSRKGm plasmid (or in the case of CtrA, CL150 carrying pSRKGm). Three biological replicates were analyzed for each strain as described in *SI Appendix*. The Affymetrix GeneChip data have been deposited under Superseries accession no. GSE124984 in the Gene Expression Omnibus database.

High-Throughput Screening of Compound Libraries. We designed a modular EGFP expression cassette, optimized for expression in *Sme* (NCBI accession no. MK387175), and constructed pSRKGm-derived plasmids containing the EGFP cassette with each *C*Las regulator gene, and candidate promoter sequences upstream of EGFP (*SI Appendix*, Table S2). Based on Affymetrix Gene Chip results for *Sme* strains ectopically expressing *C*Las regulators, candidate

target promoters were amplified and cloned upstream of the EGFP cassette (*SI Appendix*, Table S7).

Screening of strains carrying a CLas regulator and a target promoter-EGFP fusion was performed at the HTBC (http://med.stanford.edu/htbc.html). Three strains were used for screening: MB1101 pMB958 (LdtR); RFF231 pMB949 (RpoH); and MB1102 pMB956 (VisNR). Detailed screening procedures are described in *SI Appendix, Materials and Methods*.

We screened a total of 10 compound libraries (*SI Appendix*, Table S3) using the workflow is shown in *SI Appendix*, Fig. S3. Data were analyzed using MDL Assay Explorer software. A description of libraries and compound concentrations screened are given in *SI Appendix*, Tables S3–S5.

Testing Purchased Compounds. To evaluate selected results from the high-throughput screen, we purchased 10 compounds that showed inhibitory activity for 1 or more of the 3 CLas regulators: 6 of these were from the Known Bioactive Collection and 4 from the Diverse Collection (Fig. 2 and *SI Appendix*, Fig. S4). The 10 purchased compounds were also tested on each of the 3 *Sme* deletion strains ectopically expressing the corresponding *Sme* regulator (LdtR, RpoH, or VisNR) (*SI Appendix*, Table S2).

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