Examination of the Global Regulon of CsrA in *Xanthomonas citri* subsp. *citri* Using Quantitative Proteomics and Other Approaches

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The RNA-binding protein CsrA is a global posttranscriptional regulator and controls many physiological processes and virulence traits. Deletion of *csrA* caused loss of virulence, reduced motility and production of xanthan gum and substantial increase in glycogen accumulation, as well as enhanced bacterial aggregation and cell adhesion in *Xanthomonas* spp. How CsrA controls these traits is poorly understood. In this study, an isobaric tag for relative and absolute quantitation (iTRAQ)-based proteomic analysis was conducted to compare the protein profile of wild-type strain *Xanthomonas citri* subsp. *citri* and the isogenic Δ *csrA* strain. A total of 2,374 proteins were identified, and 284 were considered to be differentially expressed proteins (DEPs), among which 151 proteins were up-regulated and 133 were down-regulated in the Δ *csrA* strain with respect to the wild-type strain. Enrichment analysis and a protein-protein interaction network analysis showed that CsrA regulates bacterial secretion systems, flagella, and xanthan gum biosynthesis. Several proteins encoded by the *gumB* operon were down-regulated, whereas proteins associated with flagellum assembly and the type IV secretion system were up-regulated in the Δ *csrA* strain relative to the Xcc306 strain. These results were confirmed by β -glucuronidase assay or Western blot. RNA secondary structure prediction and a gel-shift assay indicated that CsrA binds to the Shine-Dalgarno sequence of *virB5*. In addition, the iTRAQ analysis identified 248 DEPs that were not previously identified in transcriptome analyses. Among them, CsrA regulates levels of eight regulatory proteins

(ColR, GacA, GlpR, KdgR, MoxR, PilH, RecX, and YgiX), seven TonB-dependent receptors, four outer membrane proteins, and two ferric enterobactin receptors. Taken together, this study greatly expands understanding of the regulatory network of CsrA in *X. citri* subsp. *citri*.

Keywords: citrus, CsrA, iTRAQ, posttranscriptional regulation, T4SS, virB5, *Xanthomonas citri* subsp. *citri*

Posttranscriptional regulation plays an important role in coordinating various traits, which helps bacteria rapidly respond to the changing environment (Babitzke et al. 2019). Csr (carbon storage regulator) or Rsm (repressor of stationary-phase metabolites) systems are involved in posttranscriptional regulation in Gammaproteobacteria and others (Romeo et al. 2013). CsrA/RsmA (hereafter CsrA) is a global posttranscriptional regulator and controls many physiological processes and virulence-associated traits, including central carbon metabolism, stress response, secretion system biogenesis, cell motility, biofilm formation, quorum sensing, production of extracellular products, amylase, and siderophore production (Vakulskas et al. 2015; Tang et al. 2021).

The regulatory mechanisms of CsrA have been well-documented in the model microorganism *Escherichia coli* (Pourciau et al. 2020). Its binding to RNA may cause changes in, singly or in combination, RNA secondary structure, RNA stability, translation, or Rho-mediated transcription termination (Romeo and Babitzke 2018). CsrA-mediated translational repression typically involves CsrA binding to either the 5' untranslated region (5' UTR), the initially translated region of target transcripts, or both. Overlap between a CsrA-binding site and the ribosome binding site (RBS) sequence blocks 30S ribosome access to the messenger RNA (mRNA) and inhibits translation (Babitzke and Romeo 2007). The canonical CsrA binding site often consists of a conserved GGA-containing sequence located in a hairpin loop, although CsrA also recognizes unstructured RNA (Dubey et al. 2005; Duss et al. 2014). In the last five years, a series of transcriptomic studies, including the application of crosslinking and immunoprecipitation followed by sequencing, have revealed that CsrA-binding transcripts vary substantially in different bacterial species (Dugar et al. 2016;

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Holmqvist et al. 2016; Potts et al. 2017; Sahr et al. 2017; Sowa et al. 2017).

The activity of CsrA is regulated in various modes. Small RNAs (sRNAs) that bind tightly to CsrA and antagonize its activity are a common feature of Csr/Rsm systems. Multiple sRNAs have been identified in Gammaproteobacterial species, such as CsrB and CsrC from *E. coli* (Weilbacher et al. 2003) and RsmY and RsmZ from *Pseudomonas aeruginosa* (Kay et al. 2006). Transcription of these regulatory sRNA, in return, is controlled by two-component systems, such as BarA-UvrY or GacS-GacA (Vakulskas et al. 2015), stringent response regulators (p)ppGpp and DksA (Edwards et al. 2011), and other regulators. Turnover of these RNAs in some species is regulated by the protein CsrD, which responds to preferred carbon sources (Leng et al. 2016; Suzuki et al. 2006; Vakulskas et al. 2016). In addition to regulatory sRNAs, protein antagonists of CsrA were also identified in some species, such as FliW in *Bacillus subtilis* (Mukherjee et al. 2011) and CesT in enteropathogenic *E. coli* (Katsowich et al. 2017). Expression of *csrA* itself is also regulated (Yakhnin et al. 2011). Complex regulatory circuitry of the Csr system enables bacteria to exert precise and rapid control of CsrA levels and activity.

Significant progress has been made in elucidating the regulatory roles of Csr/Rsm systems in host-pathogen interactions, including regulating bacterial colonization factors and secretion systems (Kusmieriek and Dersch 2018; Schulmeyer and Yahr 2017). However, the direct virulence-relevant targets of CsrA vary in different bacterial pathogens (Vakulskas et al. 2015). CsrA plays important roles in regulating virulence traits of *Xanthomonas* spp., which are important plant-pathogenic bacteria that cause diseases in hundreds of crops, including citrus canker caused by *Xanthomonas citri* subsp. *citri* (An et al. 2020; Ference et al. 2018). Citrus canker is a devastating disease that infects most commercial citrus varieties (Favaro et al. 2020; Ference et al. 2020). The most important virulence factors of *X. citri* subsp. *citri* are the type III secretion system (T3SS) and effectors secreted by the T3SS, such as PthA4 (Yan and Wang 2012). PthA4 is a transcription activator-like effector that activates the citrus canker susceptibility gene *LOB1* to cause the hypertrophy and hyperplasia symptoms (Duan et al. 2018; Hu et al. 2014; Jia and Wang 2020; Jia et al. 2017; Zhang et al. 2017). In addition, many other virulence-associated traits, such as quorum sensing (Li et al. 2019), extracellular polysaccharides (EPS, e.g., xanthan gum) (Rigano et al. 2007), lipopolysaccharide (LPS) (Li and Wang, 2012; Yan et al. 2012), biofilm (Li and Wang 2011), and periplasmic proteins (Zhou et al. 2015), also contribute to *X. citri* subsp. *citri* infection and colonization as well as the development of canker symptoms. These virulence traits are regulated in response to cues from the host and environment via complex regulatory networks that include factors such as HrpG/HrpX, CsrA, YtrA, Lon, TfmR, and DksA (Andrade et al. 2014; Guo et al. 2011; Teper et al. 2019; Zhang et al. 2019a; Zhou et al. 2017, 2018). In the genus *Xanthomonas*, the *csrA* deletion mutant showed nearly complete loss of virulence, reduced motility and production of EPS, substantial increase in glycogen accumulation, as well as an enhanced bacterial aggregation and cell adhesion (Chao et al. 2008; Zhu et al. 2011). Biofilm formation and dispersal was modulated by CsrA in an indirect manner correlated with cyclic di-GMP and the Clp transcription factor (Lu et al. 2012). CsrA activates the T3SS by stabilizing the 5' UTR of *hrpG*, the master virulence regulator (Andrade et al. 2014; Guo et al. 2011). Recently, CsrA of *X. citri* subsp. *citri* was found to control the type IV secretion system (T4SS) homeostasis by inhibiting the translation initiation of *virB7* (Cenens et al. 2020).

The regulatory circuitry of the Csr/Rsm system in *Xanthomonas* remains poorly understood. First, CsrA-binding sRNAs

similar to CsrB/C have not yet been identified. Intriguingly, a genome-wide screening in *X. campestris* pv. *campestris* revealed a novel RNA that sequesters CsrA, which does not belong to the CsrB/C family (Tang et al. 2020). Second, environmental cues and upstream regulators of *csrA* expression have yet to be identified. Finally, the direct targets of CsrA in *Xanthomonas* spp. remain largely unknown. We hypothesized that CsrA regulates direct targets beyond *hrpG* and *virB7* in *Xanthomonas* spp. Here, an isobaric tag for relative and absolute quantitation (iTRAQ)-based proteomic study was used to identify the proteins that are regulated by CsrA in *X. citri* subsp. *citri*. Proteomic study has been widely used in the study of different pathosystems (Cui et al. 2020; Wang et al. 2021; Yan et al. 2021). The putative targets were further verified and their roles were assessed using a variety of approaches. The results of this study greatly expand understanding of the regulatory network of CsrA in the genus *Xanthomonas*.

RESULTS

Proteome profiling of Xcc306 and isogenic Δ csrA strains.

To investigate the global roles of CsrA in *X. citri* subsp. *citri*, iTRAQ was applied to generate protein profiles of the wild-type strain Xcc306 and its isogenic Δ csrA mutant cultured in *hrp*-inducing medium XVM2. A total of 2,374 proteins were identified by iTRAQ (Supplementary Table S1), which account for about 53.6% of all protein-coding genes of Xcc306. Proteins with a ratio (Δ csrA/WT) <0.77 or >1.3 and a *P* value <0.05 were considered as differentially expressed proteins (DEPs); 284 proteins were differentially expressed, including 151 upregulated and 133 downregulated proteins (Supplementary Table S2).

Comparison and enrichment analyses were performed using the iTRAQ data as well as transcriptomic data obtained previously by our lab (Andrade et al. 2014). A Venn diagram showed that expression effects of CsrA on 36 genes overlapped between the transcription and protein levels (Fig. 1A; Table 1). Further analysis classified the 36 genes into several functional categories, including chemotaxis and flagellum biosynthesis, T3SS and effector genes, T4SS, enzymes, and others (Table 1). Although the comparison of proteomic data and transcriptomic data displayed considerable differences with respect to CsrA regulation, enrichment analysis based on the COG (Clusters of Orthologous Groups of proteins) database showed that cell motility and intracellular trafficking and secretion are over-represented categories for both proteomic and microarray data (Table 2). The group of signal transduction mechanisms was enriched only for microarray data (Table 2), which may be associated with technical challenges in extraction and solubilization of membrane-associated signaling proteins (Walls and Loughran 2011). In addition, similar results were obtained from Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis using clusterProfiler (v3.16.1) (Yu et al. 2012). Based on iTRAQ data, pathways involved in flagellum assembly and bacterial secretion system were especially over-represented (Fig. 1B).

Protein-protein interaction (PPI) analysis of the CsrA regulon.

To reveal the regulon of CsrA and the putative protein-protein association network, the protein-protein association data of Xcc306 was derived from the STRING database (Marees et al. 2018). The interaction data for all of the DEPs with an interaction score ≥ 0.50 were extracted (Supplementary Table S3). The protein-protein association network was generated using software Cytoscape (v3.8.0) (Shannon et al. 2003). Three separate gene clusters were shown in circular layout and the closely correlated genes appeared to demonstrate similar trends at the protein level (Fig. 2). Apparently, most T4SS structural

Fig. 1. Profiling the proteomes of *Xcc306* and Δ *csrA*. **A**, Venn diagram of differentially expressed genes identified by isobaric tags for relative and absolute quantitation (iTRAQ) and microarray. **B**, Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis of differentially expressed proteins derived from iTRAQ data. The enrichment analysis was performed using R package clusterProfiler (v3.16.1). p.adjust represents adjusted *P* value for multiple comparisons calculated by the Benjamini and Hochberg method (Benjamini and Hochberg 1995).

Table 1. Differentially expressed genes identified by both isobaric tags for relative and absolute quantitation (iTRAQ) and microarray analyses

Functional categories, locus tag	Gene	Protein product	Fold change ^a	
			ΔcsrA/WT	ΔcsrA/WT
Chemotaxis and flagellar biosynthesis				
XAC1930	<i>cheA</i>	Chemotaxis protein	2.36	2.68
XAC1931	<i>cheZ</i>	Chemotaxis protein	2.86	4.76
XAC1933	<i>fliA</i>	RNA polymerase sigma factor	2.66	5.03
XAC1934	<i>fleN</i>	Flagellar biosynthesis switch protein	2.16	6.59
XAC1946	<i>fliN</i>	Flagellar protein	2.74	4.89
XAC1947	<i>fliM</i>	Flagellar motor switch protein FliM	2.38	4.06
XAC1948	<i>fliL</i>	Flagellar protein	2.39	3.56
XAC1949	<i>fliK</i>	Flagellar protein	1.60	3.27
XAC1954	<i>fliF</i>	Flagellar MS-ring protein	2.50	2.95
XAC1975	<i>fliC</i>	Flagellin	3.16	2.85
XAC1976	<i>flgL</i>	Flagellar protein	1.94	2.79
XAC1983	<i>flgE</i>	Flagellar hook protein FlgE	4.02	2.97
XAC1932	<i>cheY</i>	Chemotaxis protein	2.87	5.43
Type III secretion system				
XAC0076	<i>avrBS2</i>	T3S effector	0.73	0.50
XAC1172	<i>xopAV</i>	T3S effector	0.68	0.40
Type IV secretion system				
XAC2614	<i>virB4</i>	VirB4 protein	3.23	2.87
XAC2618	<i>virB11</i>	VirB11 protein	3.82	5.24
XAC2619	<i>virB10</i>	VirB10 protein	3.98	3.94
Enzyme				
XAC0346	—	Degenerated cellulase	2.10	4.32
XAC0466	—	Lytic enzyme	3.53	3.12
XAC1200	—	Prolyl oligopeptidase	2.20	2.07
XAC3031	—	Histidine kinase	2.15	2.89
XAC2885	—	Phospholipase A1	2.61	3.63
XAC2609	—	Carboxypeptidase	5.00	7.01
Hypothetical protein				
XAC0467	—	Hypothetical protein	3.23	2.17
XAC1145	—	Hypothetical protein	2.70	2.17
XAC3267	—	Hypothetical protein	2.30	2.17
XAC3279	—	Hypothetical protein	4.92	5.24
XAC3634	—	Hypothetical protein	5.34	3.05
XAC1397	—	Hypothetical protein	2.88	2.91
XAC2610	—	Hypothetical protein	5.31	4.69
XAC2443	—	Hypothetical protein	1.52	2.69
Others				
XAC0868	—	Uncharacterized protein	3.24	3.63
XAC2529	<i>rhsD</i>	RhsD protein	2.00	3.29
XAC1907	<i>parA</i>	Chromosome partitioning protein	1.89	4.59
XAC1573	<i>phoU</i>	Phosphate regulon transcriptional regulator	1.86	6.28

^a iTRAQ data and microarray data, respectively.

proteins were up-regulated in the $\Delta csrA$ strain compared with the Xcc306 strain (Fig. 2A). Also, many flagellum assembly proteins and some pilus assembly proteins were up-regulated in the $\Delta csrA$ strain (Fig. 2B). However, several proteins involved in xanthan gum biosynthesis were down-regulated in the $\Delta csrA$ strain compared with that of Xcc306 (Fig. 2C). Taken together, the biological network displays of the CsrA regulon in *X. citri* subsp. *citri* suggested a pivotal role of CsrA in T4SS, flagellum assembly, and xanthan gum biosynthesis.

CsrA positively regulates the gum gene cluster.

Xanthan gum is an EPS that contributes to biofilm formation, epiphytic fitness, and tolerance to environmental stress in *Xanthomonas* spp. (An et al. 2020; Büttner and Bonas 2010). Xanthan gum biosynthesis is mediated by the gum operon, which is composed of 12 enzyme-coding genes (*gumB* to *gumM*) (Vojnov et al. 2001). Although microarray data did not show that CsrA affects the expression of a gum gene cluster at the transcription level (Andrade et al. 2014), our proteomic analysis displayed that nine of 12 gum biosynthesis genes were down-regulated in the $\Delta csrA$ strain compared with those of the wild-type strain Xcc306 (Fig. 3A). To further confirm this result, a reporter assay was applied to test the expression of *gumB*, the first gene of this operon. β -Glucuronidase (GUS) assays using translational fusion plasmids showed that expression of *gumB* in the $\Delta csrA$ strain was substantially lower than in the wild-type strain Xcc306 (Fig. 3B). Because a translational fusion measures both transcriptional and translation effects and our microarray data showed no effect, we inferred that CsrA positively regulates *gumB* and, most likely, other *gum* genes at the translational level.

CsrA negatively regulates flagellum assembly genes.

In the *Xanthomonas* genus, over 40 genes are involved in flagellum biogenesis and chemotaxis. Furthermore, expression of flagellum structural genes is regulated by several regulators in a hierarchical manner, including transcriptional regulator FleQ and RNA polymerase sigma factor FliA (Yang et al. 2009).

Genomic organization of *X. citri* subsp. *citri* flagellum genes spans from gene *cheA* (XAC1930) to gene *flgM* (XAC1989), many of which were regulated by CsrA at the protein or mRNA level (Fig. 4A). Furthermore, according to KEGG pathway analysis, 37 genes are associated with flagellum assembly in Xcc306, and expression data for these flagellum genes are listed in Supplementary Table S4. Among them, 14 and 20 of these genes were upregulated in $\Delta csrA$ at protein and mRNA levels, respectively (Fig. 4B). Altogether, nine of 37 flagellum assembly genes exhibited similar regulatory effects at both the transcriptional and translational levels (Fig. 4B). To further confirm the expression changes from iTRAQ data, a hemagglutinin (HA) tag was inserted in-frame at the end of *flgQ*- and *fliA*-coding sequences of Xcc306 and the $\Delta csrA$ strain. As expected, Western blot using anti-HA showed that FliA expression rather than FleQ expression is induced in $\Delta csrA$ relative to Xcc306 (Fig. 4C). To summarize, these data indicate that CsrA of *X. citri* subsp. *citri* negatively regulates expression of the flagellar proteins.

CsrA negatively regulates T4SS and its effectors.

T4SS in *X. citri* subsp. *citri* is composed of a set of 12 core proteins, i.e., VirB1 through B11 plus VirD4, enabling *X. citri* subsp. *citri* to kill other competitive bacterial species by the secretion of T4SS effectors (toxins) (Souza et al. 2015). Our proteomic data show that most T4SS structural proteins as well as its effector protein (X-Tfe) were upregulated in $\Delta csrA$ compared with that of wild-type strain Xcc306 (Fig. 5A). To further confirm this, a GUS assay was performed using a translational fusion construct. Consistent with proteomic data, GUS activity of *virD4* in $\Delta csrA$ strain was much higher than that of wild-type strain Xcc306 (Fig. 5B). In addition, some putative type IV effectors (XAC3634, XAC2609, XAC0466, XAC0096, XAC2885, XAC1918, XAC3266) as well as their putative proximal immunity proteins (XAC3633, XAC2610, XAC0467, XAC0097, XAC2884) were up-regulated at the protein level or at the mRNA level in the $\Delta csrA$ strain compared with the wild-type strain (Supplementary Table S5).

Table 2. Functional enrichment analysis based on Clusters of Orthologous Groups database

Functional classification	Abbreviation	P value	
		Microarray	iTRAQ ^b
RNA processing and modification	A	1.00	1.00
Chromatin structure and dynamics	B	1.00	1.00
Energy production and conversion	C	0.02	0.89
Cell cycle control, mitosis and meiosis	D	0.71	0.77
Amino acid transport and metabolism	E	0.43	0.27
Nucleotide transport and metabolism	F	0.26	0.28
Carbohydrate transport and metabolism	G	0.40	0.52
Coenzyme transport and metabolism	H	0.18	0.38
Lipid transport and metabolism	I	0.07	0.43
Translation, ribosomal structure and biogenesis	J	0.01	0.10
Transcription	K	0.75	0.61
Replication, recombination, and repair	L	0.01	0.02
Cell wall/membrane biogenesis	M	1.00	0.18
Cell motility	N	8.46E-18	3.86E-06
Posttranslational modification, protein turnover, chaperones	O	0.83	0.62
Inorganic ion transport and metabolism	P	0.31	1.00
Secondary metabolites biosynthesis, transport and catabolism	Q	1.00	1.00
Function unknown	S	0.54	0.69
Signal transduction mechanisms	T	0.03	0.79
Intracellular trafficking and secretion	U	1.18E-06	6.68E-03
Defense mechanisms	V	0.52	0.44
Extracellular structures	W	1.00	1.00
Cytoskeleton	Z	1.00	1.00

^a iTRAQ = isobaric tags for relative and absolute quantitation data. P values were calculated by Fisher exact test. Bold indicates an over-represented group (P value < 0.05).

Interestingly, four T4SS-associated proteins (X-Tfe^{XAC2609}, X-Tfi^{XAC2610}, VirB5^{XAC2613}, VirB9^{XAC2620}) belong to the top 10 upregulated proteins in the Δ *csrA* strain compared with those of the wild-type strain Xcc306 (Fig. 5C). Based on the features of the CsrA-binding motif (Duss et al. 2014), DNA sequence analyses to search for potential CsrA-binding motifs were conducted for all genes in the *VirB* operon. Secondary mRNA structure analyses by Mfold (Zuker 2003) revealed one potential CsrA-binding target close to the translation start site of *virB5*.

The region of *virB5* is predicted to form a hairpin structure with a hexa-loop composed of the conserved motif ANGGAX, suggesting that *virB5* may be a putative CsrA-binding target (Fig. 5D). To further explore this, an electrophoretic mobility-shift assay (EMSA) was conducted, which showed that CsrA binds to the region of *virB5* (Fig. 5E and F). Mutation of the predicted CsrA-binding site GGA (Fig. 5D) to TTA abolished the mobility shift caused by CsrA (Fig. 5F). As suspected, the CsrA-binding site overlaps with the Shine-Dalgarno sequence of

Fig. 2. Visualization of protein-protein association network for differentially expressed proteins (DEPs). All 284 DEPs were checked for putative interaction based on the STRING (v11) database. DEPs with the interaction score ≥ 0.50 were extracted and imported into software Cytoscape (v3.8.0). Protein expression data and the interaction scores were used to color the nodes and the edges of this biological network, respectively. Only three striking clusters of interacting nodes (of 17 total) are shown here. **A**, Cluster of structural proteins involved in the type IV secretion system. **B**, Cluster of structural proteins involved in flagellar and pilus assembly. **C**, Cluster of proteins involved in xanthan gum biosynthesis.

the RBS of *virB5*, which suggests that the binding of CsrA can prevent the docking of 30S ribosomal subunit, thus mediating translational repression.

CsrA plays a role in motility, exopolysaccharide production, and biofilm formation.

To verify the data obtained in our comparative proteome analysis, we tested the regulation of CsrA on motility, EPS production, and biofilm formation. We compared the motility of wild-type and $\Delta csrA$ mutant strains and observed that deletion of *csrA* impaired motility in the tested conditions (Fig. 6A and B; Supplementary Fig. S1). Interestingly, a previous study demonstrated that motility in *X. citri* subsp. *citri* depends on EPS production (Gottig et al. 2009). In accordance with this report, mutation in *gumD*, which encodes an essential protein involved in xanthan gum biosynthesis, caused a reduction of the motility (Fig. 6A and B). Complementation of the *csrA* mutant restored the motility phenotype to that observed in the wild-type strain (Fig. 6A and B). These results indicated that CsrA may positively regulate the xanthan gum production. We further quantified the EPS production in supernatants of *X. citri* subsp. *citri* strains and observed that mutation of *csrA* decreased exopolysaccharide production by approximately threefold, whereas mutation of *gumD* reduced approximately fourfold compared with wild-type (Fig. 6C). The $\Delta csrA$ complemented strain was

able to restore the exopolysaccharide production to similar levels as in the wild-type strain. We also evaluated whether *csrA* is involved in regulation of biofilm formation in *X. citri* subsp. *citri*. Interestingly, the $\Delta csrA$ strain showed higher biofilm formation on a glass surface than the wild-type strain, whereas the complementation strain was similar to that of the wild-type (Fig. 6D).

DISCUSSION

Translation repression and activation are two modes of CsrA regulation that enable bacteria to control gene expression and rapidly respond to external stimuli (Vakulskas et al. 2015). Our iTRAQ data revealed both positive and negative regulation by CsrA. While this is consistent with these two presumed modes of action, many of the observed regulatory effects may be caused indirectly, because CsrA affected the expression of numerous regulatory genes (Supplementary Table S2). We have further expanded knowledge of the target proteins that are under direct or indirect regulation by CsrA, which helps to explain the multiple physiological and phenotypical changes caused by the knockout of CsrA in *Xanthomonas* spp. Here, we demonstrated that CsrA regulates genes involved in xanthan gum biosynthesis, flagellum assembly and T4SS, and beyond. This study expands the regulatory network of CsrA and leads to the discovery of a novel CsrA-binding target RNA in *X. citri* subsp. *citri*.

Fig. 3. CsrA positively regulates the gum gene cluster. **A**, Genomic organization of the xanthan gum gene cluster and its expression at the protein level in the $\Delta csrA$ strain compared with the wild-type strain. NI = proteins that are not identified by isobaric tags for relative and absolute quantitation. **B**, Expression of *gumB* in the Xcc306 and $\Delta csrA$ strains by β -glucuronidase (GUS) assay. *gyrA* was used as an endogenous control. Values represent means $\pm$ standard deviation and asterisks indicate statistical significance, using Student's *t* test ($P < 0.05$, $n = 3$). This experiment was repeated twice in triplicates with similar results.

A characteristic feature of xanthomonads is xanthan gum, which confers a mucoid appearance to the bacterial colonies (An et al. 2020; Büttner and Bonas 2010). Xanthan gum is not required for full pathogenicity of *X. citri* subsp. *citri* but contributes to its epiphytic survival and initial infection (Dunger et al. 2007; Li and Wang 2014). Xanthan gum is an important component of biofilms, which facilitate the formation of bacterial aggregates and proliferation in the intercellular space apoplast, and subsequent symptom development (Li and Wang 2011; Rigano et al. 2007). Deletion of *csrA* of *X. citri* subsp. *citri* caused significant reduction in production of EPS, which is consistent with previous studies, although gene expression change of *gumB* at the transcriptional level was not observed (Chao et al. 2008; Zhu et al. 2011). Here, our iTRAQ data clearly showed that CsrA positively controls the expression of the *gumB* operon at a translational level via an unknown mechanism. In addition to the CAP-like protein (Chen et al. 2010), the GntR family regulator HpaR1 (Su et al. 2016), and the transcriptional regulator CysB (Schulte et al. 2019), CsrA appears to be another regulator that controls xanthan gum biosynthesis in *Xanthomonas* genus. Intriguingly, CsrA was suggested to positively control exopolysaccharide amylovoran production, partly by repressing Lon protease, in *Erwinia amylovora* (Lee et al. 2018; Zhou et al. 2018). It remains to be determined whether a similar mechanism exists in *X. citri* subsp. *citri*.

Although the working modes vary in different species, it seems to be a common theme that CsrA regulates bacterial flagellum synthesis and motility. In *E. coli*, CsrA stabilizes the mRNA of the master regulatory genes *flhDC* and, therefore,

positively regulates flagellum biosynthesis (Wei et al. 2001; Yakhnin et al. 2013). In *Bacillus subtilis*, CsrA represses translation of the flagellin gene *hag* and interaction of FlhW protein with CsrA inhibits this activity. This circuitry is essential to flagellin homeostasis and flagellar morphogenesis (Mukherjee et al. 2011; Yakhnin et al. 2007). In *Legionella pneumophila*, CsrA directly targets the mRNAs of two regulatory genes, *fleQ* and *fleR*, and inhibits their translation (Sahr et al. 2017). Intriguingly, in *X. citri* subsp. *citri*, the master regulatory gene *fleQ* was not altered either at the mRNA or protein levels in the $\Delta csrA$ strain compared with that of wild-type strain Xcc306. CsrA positively regulates PilH but negatively regulates the expression of most pili genes. It was reported that PilH negatively regulates flagellum-dependent swimming but positively regulates pili-dependent swarming motility in *X. campestris* pv. *campestris* (Qi et al. 2020). The mechanistic understanding of how CsrA regulates flagellar and pili genes needs further elucidation. The $\Delta csrA$ mutation in *Xanthomonas* spp. causes the loss of motility but also leads to an increase in expression of flagellum genes. This observation might be partially explained by the significant reduction in xanthan gum, since it provides an appropriate fluid microenvironment for motility in specific conditions (Harshey 2003). Consistent with this reasoning, mutation of *gumD* abolished the motility of *X. citri* subsp. *citri* in the motility assay. In addition, the *csrA* mutant appears to be more sticky on the glass wall than the wild-type *X. citri* subsp. *citri*, which might further explain the effect of CsrA on controlling both motility and sessile behavior. Moreover, the effect of CsrA on regulating other factors in *X. citri* subsp. *citri* that may also be

Fig. 4. CsrA negatively regulates flagellum assembly genes. **A**, Genomic organization and gene expression of flagellum assembly genes in the $\Delta csrA$ strain compared with the wild-type strain. Upregulated genes at both the transcriptional and protein level are in blue, genes upregulated only at protein level are in green, genes upregulated only at transcriptional level are in red, and genes not changed at transcriptional or protein level are in black. Asterisks indicate master regulatory genes. **B**, Venn diagram of upregulated flagellar assembly genes revealed by isobaric tags for relative and absolute quantitation and microarray. **C**, Quantification of FleQ and FliA by Western blot. GroEL was used as an endogenous control. Relative band intensities were quantified by ImageJ (v1.52) and are shown at the bottom of each band.

Fig. 5. CsrA negatively regulates the type IV secretion system (T4SS) and its effectors. **A**, Genomic organization of the T4SS gene cluster and its expression changes at protein level. NI = not identified by isobaric tags for relative and absolute quantitation; X-Tfe = *Xanthomonadaceae* T4SS effector; X-Tfi = *Xanthomonadaceae* T4SS immunity protein. **B**, Expression of *virD4* in Xcc306 and $\Delta csrA$ strains by β -glucuronidase (GUS) assay. Values represent means $\pm$ standard deviation and asterisks indicate statistical significance, using Student's *t* test ($P < 0.05$, $n = 3$). **C**, Overview of differentially expressed proteins (DEPs) by volcano plot. Four T4SS-associated genes of interest are annotated. **D**, Secondary structure prediction of the potential target region of *virB5*. A typical CsrA-binding site is shown inside the box. +1 = translational start site (TTS). **E**, Gel-shift assay for interaction between CsrA protein and the potential target region of *virB5*. The length of *virB5* transcript is 118 nt. The 126-nt transcript from the *hrpX* coding region was used as a negative control. **F**, RNA mobility shift assays for interaction between CsrA protein and wild type and mutated *virB5* RNA probe. The *virB5*-TTA transcript was constructed by mutagenizing the putative binding site GGA to TTA.

involved in flagellum biogenesis and function needs to be further investigated.

It only recently has been revealed that CsrA contributes to homeostasis of the bactericidal T4SS by maintaining a constant though partial repression of the *virB* operon via direct translation inhibition of *virB7* (Cenens et al. 2020). Consistent with the previous study, our proteomic data showed that knockout of *csrA* caused a substantial upregulation of most genes of the *virB* operon. Interestingly, *virB5* was the most upregulated gene of the *virB* operon. RNA secondary structure prediction demonstrated that the untranslated leader sequence of *virB5* contains the features of a typical CsrA-binding motif. Furthermore, a gel-shift assay verified the interaction between CsrA and the leader sequence of *virB5*. Unlike *virB7*, *virB5* is located in the middle of the *virB* operon. Similarly, it has been reported that CsrA can act on an internal gene of an operon (Pannuri et al. 2012) or more than one target of the same pathway (Andrade et al. 2014). Our results thereby indicate that CsrA negatively affects the levels of the T4SS-associated proteins by directly inhibiting the translation of both *virB7* and *virB5*.

Importantly, the iTRAQ analysis identified 248 DEPs that were not identified by transcriptome analysis (Andrade et al. 2014). This is almost certainly because iTRAQ identifies CsrA targets at the

protein level, whereas microarray analysis detects targets at the mRNA level. The iTRAQ data suggest that CsrA regulates eight transcriptional regulators (ColR, GacA, GlpR, KdgR, MoxR, PilH, RecX, and YgiX) that further expand its regulatory spectrum and are predicted to contribute to its broad regulatory effects on numerous physiological processes and virulence traits in *Xanthomonas* spp. Among them, ColR plays multiple roles in the pathogenicity of *X. citri* subsp. *citri*, including regulating multiple *hrp* genes, biofilm formation, LPS production, and catalase activity (Yan and Wang 2011). GacA is involved in chemotaxis but does not have a significant effect on pathogenicity in *Xanthomonas* spp. (Yang et al. 2007). KdgR is an IclR family transcriptional regulator that inhibits virulence mainly by repressing expression of *hrp* genes in *X. oryzae* pv. *oryzae* (Lu et al. 2011). Interestingly, CsrA is under positive control of KdgR in *Erwinia carotovora* subsp. *carotovora* (Hyytiäinen et al. 2001), indicating a complex regulatory system between them. RecX inhibits both RecA recombinase and coprotease activities through direct physical interaction (Pagès et al. 2003). The mechanistic understanding of the interactions between CsrA and these regulators is largely unknown and needs further investigation. In addition, CsrA regulates the levels of seven TonB-dependent receptors, four outer membrane proteins, two ferric enterobactin receptors, and many other gene products

Fig. 6. CsrA regulates the motility, extracellular polysaccharides (EPS) production and biofilm formation in *Xanthomonas citri* subsp. *citri*. **A**, Motility assay on 0.5% Silva Buddenhagen (SB) agar plates. Cells were spotted and experiments were analyzed after 48 h of incubation at 28°C. **B**, Measurements of the diameters of cell spread on plates after 48 h of incubation at 28°C. Values are expressed as the means $\pm$ standard deviation of four independent biological replicates. **C**, EPS production by *X. citri* subsp. *citri* strains. Mean values $\pm$ standard deviation derived from two independent experiments with three biological replicates each are shown. **D**, Biofilm formation of *X. citri* subsp. *citri* strains on a glass surface was assessed. Cells were grown in rich medium at 28°C for 72 h, then biofilms were stained by crystal violet (CV) and were solubilized in 30% acetic acid. Ratio A (595/600 nm) represents the CV quantification and normalization by culture optical density. The means of three biological replicates and standard deviation values are shown. An asterisk (*) indicates significant difference based on one-way analysis of variance with Bonferroni ($P < 0.001$) compared with the wild-type *X. citri* subsp. *citri* strain (WT), Δ csmD and *csmD* mutant strain, Δ csrA and *csrA* mutant strain carrying the empty vector, and Δ csrA + pcsrA and complemented strain.

that span a broad spectrum of features, which need further characterization. Intriguingly, altered trends in expression of seven *hrp* structural genes were identified by iTRAQ, with only one gene, *HrpF*, having a *P* value <0.05, whereas 24 *hrp* structural genes were identified to be differentially regulated by CsrA in the microarray data (Andrade et al. 2014). This likely results from limited sensitivity of iTRAQ (Bachor et al. 2019), which needs to be taken into consideration for future studies.

In summary, we found that CsrA regulates the levels of proteins involved in xanthan gum biosynthesis, flagellum assembly, and T4SS biogenesis and function. CsrA is also known to positively regulate T3SS and its effectors by stabilizing the transcript of *hrpG*. This study likewise identified many new targets of CsrA that may give rise to new research questions that further enhance our understanding of the vital function of CsrA.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions.

Strains and plasmids used in this study are listed in Table 3. *E. coli* cells were cultured in lysogeny broth medium at 37°C. *Xanthomonas* strains were cultured in nutrient broth (NB) medium and on nutrient agar (NA) plates at 28°C. For induction of *hrp* genes, *X. citri* subsp. *citri* strains were grown in XVM2 medium (Wengelnik and Bonas 1996). When required, media were supplemented with antibiotics as follows: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; or gentamycin, 10 µg/ml.

Oligonucleotides used are listed in the Supplementary Table S6 and restriction digestion and DNA ligation were performed in accordance with manufacturer instructions (New England Biolabs). To construct the *gumD* deletion mutant, approximately 1 kb of the upstream and downstream regions of the target gene

were amplified by PCR from *X. citri* subsp. *citri* genomic DNA and the two fragments were fused by PCR, to produce an in-frame deletion (Andrade et al. 2014). The generated fragment was then cloned into the *EcoRI* restriction site of the pNPTS138 suicide vector, generating the construct pNPTS-*gumD* (Table 3). The construct was introduced into *X. citri* subsp. *citri* by electroporation, and the wild-type *gumD* was replaced by the deleted version after two recombination events, as described previously (Andrade, et al. 2014). The *gumD* deletion mutant were confirmed by PCR.

iTRAQ and identification of DEPs.

iTRAQ procedures were conducted as previously described (Klee et al. 2018). Briefly, the wild-type strain Xcc306 and its Δ *csrA* derivative (four biological replicates per strain) were cultured in defined XVM2 medium and were harvested at the exponential phase of growth (optical density at 600 nm [OD₆₀₀] = 0.35). The proteins (100 µg) from each sample were digested with trypsin and were labeled with 8-plex isobaric tags. Wild-type replicates were labeled with 113,114, 115, and 116 tags, and Δ *csrA* replicates with 117, 118, 119, and 121 tags. Labeled peptides were combined and further fractionated, using SCX chromatography on an Agilent HPLC system 1100. Peptide analyses on the Q-Exact Plus Mass Spectrometer (Thermo Fisher Scientific) were conducted at the Interdisciplinary Center for Biotechnology Research of the University of Florida. Protein identification and quantification were carried out, using Max-Quant (v1.6), against the proteome of Xcc306 downloaded from UniProt database (Tyanova et al. 2016). Proteins with a ratio (Δ *csrA*/WT) <0.77 or >1.3 and *P* value <0.05 by student's *t* test were considered to be DEPs.

Table 3. Strains and plasmids used in this study^a

Types/strains	Relevant characteristic	Reference or source
<i>Escherichia coli</i>		
BL21(DE3)	fluA2 [lon] ompT gal (λDE3) [dcm] ΔhsdS λ DE3 = λ sBamHI Δ <i>EcoRI</i> -B int::(<i>lacI</i> ::PlacUV5::T7 gene1) i21 Δnin5	Novagen
S-17-1 λpir	RK2 tra regulon, pir, host for pir-dependent plasmid pOK1	Simon et al. 1983
<i>Xanthomonas citri</i> subsp. <i>Citri</i>		
Xcc306	Syn. <i>X. axonopodis</i> pv. <i>citri</i> 306; wild type, Rif ^r	da Silva et al. 2002
Xcc306_fliA_HA	HA tag insertion strain in the background of Xcc306, Rif ^r	This study
Xcc306_fleQ_HA	HA tag insertion strain in the background of Xcc306, Rif ^r	This study
XccΔ <i>gumD</i>	The <i>gumD</i> deletion mutant of <i>X. citri</i> subsp. <i>citri</i>	This study
Plasmids		
pGUS	pBBR1MCS-5 derivative containing gus gene followed by T7 terminator cloned in reverse orientation of lac promoter.	Teper et al. 2019
pGUS: <i>pgyrA</i>	pGUS derivative. The 189 bp upstream region of XAC1631 was cloned upstream to gus, Gn ^R	Teper et al. 2019
pGUS: <i>pgumB</i>	pGUS derivative. The 527 bp upstream region of XAC2585 was cloned upstream to gus, Gn ^R	This study
pGUS: <i>pvirD4</i>	pGUS derivative. The 268 bp upstream region of XAC2623 was cloned upstream to gus, Gn ^R	This study
pOK1	sacB sacQ mobRK2 oriR6K, suicide vector, Sp ^R	Huguet et al. 1998
pOK1:fliA_HA	pOK1 derivative containing the <i>fliA</i> in frame with 3' HA tag, Sp ^R	This study
pOK1:fleQ_HA	pOK1 derivative containing the <i>fleQ</i> in frame with 3' HA tag, Sp ^R	This study
pET-28a:CsrA	pET-28a derivative containing <i>csrA</i> downstream T7 promoter, Kn ^R	Andrade et al. 2014
pNPTS- <i>gumD</i>	The suicide vector pNPTS138 containing 1 kb of the upstream and downstream regions of <i>gumD</i> to produce an in-frame deletion mutant of <i>gumD</i>	This study
pMD19	T-vector for PCR cloning, Ap ^r	Takara
pMD19-5UTR <i>virB5</i>	5' UTR of <i>virB5</i> with T7 promoter cloned in pMD19, Ap ^r	This study
pMD19-5UTR <i>virB5</i> -TTA	5' UTR of <i>virB5</i> mutated from GGA to TTA, Ap ^r	This study

^a HA = hemagglutinin; Kn^R, Gn^R, Sp^R, Rif^R, and Ap^R indicate resistance to kanamycin, gentamicin, rifampicin, and ampicillin, respectively; UTR = untranslated region.

Bioinformatic analysis.

For enrichment analysis, a COG functional category was assigned to the protein-coding genes of *X. citri* subsp. *citri* using eggNOG-mapper (v2) (Huerta-Cepas et al. 2017). Functional enrichment analysis for either differentially expressed genes or DEPs was performed using Fisher's exact test within the R environment (v4.0.0) (Abatangelo et al. 2009). In addition, clusterProfiler (v3.16.1) was used for pathway enrichment analysis (Yu et al. 2012). For PPI analysis, all 284 DEPs were checked for putative interactions based on a STRING (v11.0) database (Szklarczyk et al. 2019). DEPs with an interaction score ≥ 0.5 were extracted. These data, including interaction scores and a gene expression table, were imported into software Cytoscape (v3.8.0) to construct biomolecular interaction networks (Shannon et al. 2003). For RNA secondary structure prediction, the software Mfold web server was used (Zuker 2003).

Western blot.

To label proteins at the native genomic loci, an HA tag (YPYDVPDYA) coding sequence was incorporated into the 3' end of *FlaA*- and *FlaQ*-coding sequence by PCR, according to the designed primers (Supplementary Table S6). For each gene, the two amplicons were combined together into one fragment, using overlap PCR. After restriction enzyme digestion, the whole fragment containing the HA tag coding sequence and its flanking regions was inserted into the suicide vector pOK1. Recombinant pOK1 plasmid was transferred to the wild-type Xcc306 and Δ *csrA* strains by electroporation, and markerless replacement mutants were produced, using a two-step sucrose counter-selection method (Zhang et al. 2019a). Wild-type strain Xcc306 and the Δ *csrA* strain containing HA-tag were cultured in XVM2 medium. At exponential phase ($OD_{600} = 0.35$), bacteria were harvested, were ruptured by sonication, and were centrifuged at 14,000 rpm for 30 min under 4°C using a centrifuge (Eppendorf). Protein extracts were quantified by the Bradford method at 595 nm, using a Bio-Rad protein assay kit (Bio-Rad). Equal amounts of proteins were loaded, were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and were transferred to a nitrocellulose membrane (GE Healthcare). The membrane was then incubated with anti-HA tag antibody (Roche), followed by the anti-immunoglobulin G horseradish peroxidase-conjugated antibody (Abcam). As a control, immunoblotting was carried out for the same protein extracts using anti-*groEL* antibody (Abcam). Immunoblots were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and were detected by C-DiGit Blot Scanner (LI-COR Biosciences).

GUS assay.

To generate GUS reporter plasmids, the putative promoter region, untranslated leader segment, plus a small portion of the coding region of the tested genes was amplified with designed primers (Supplementary Table S6) and was cloned into pGUS vector (Teper et al. 2019). Recombinant pGUS vectors were transferred into wild-type Xcc306 and Δ *csrA* strains by electroporation. GUS activity was quantified as described previously (Wang et al. 2006a and b; Zhang et al. 2019a). Briefly, bacterial cells cultured in the XVM2 medium were harvested and were resuspended in PBS buffer. After sonication, 20 μ l of clear supernatant was added to 80 μ l of phosphate buffered saline (PBS) containing 1.25 μ M p-nitrophenyl β -D-glucopyranoside in a 96-well plate. Stop buffer (100 μ l of 0.4 M Na_2CO_3) was added to stop the reaction immediately when yellow color appeared during incubation at 37°C and the reaction time was also recorded. The absorbance of reaction solution was measured at 405 nm and protein amount was calculated by the Bradford method at 595 nm, using a Bio-Rad protein assay kit (Bio-

Rad). GUS activity was quantified by arbitrary units and was determined as $A_{405}/(\text{time in min} \times A_{595} \times 0.02)$. The experiment was repeated at least twice in triplicates with similar results.

In vitro transcription.

Briefly, to produce the DNA templates for the targeted mRNA transcripts, two rounds of PCR amplification were performed to fuse the T7 promoter sequence with the DNA sequence for the targeted mRNA transcripts, using the specific primer pairs (Supplementary Table S6). The transcripts of *hrpX* and *virB5* were produced in vitro using the SP6/T7 transcription kit (Roche) and Biotin RNA labeling kit (Roche), following manufacturer instructions.

To generate the mutated *virB5*-TTA transcript, the 5' UTR of *virB5* was cloned into pMD19 T-vector and the predicted GGA binding site was changed to TTA, using a Q5 site-directed mutagenesis kit (New England Biolabs), which was used for in vitro transcription.

All transcripts were purified using the ZymoClean gel RNA recovery kit (Zymo Research) and were resuspended in diethyl pyrocarbonate water. RNA product was quantified with NanoDrop (NanoDrop Technologies).

Purification of CsrA.

The purification of CsrA was described previously (Andrade et al. 2014). Briefly, the recombinant 6His-CsrA protein was overexpressed in *E. coli* BL21 (DE3) (Novagen) by the addition of 1 mM isopropyl- β -D-thiogalactopyranoside to cells at mid-exponential phase. Bacterial cells were harvested 4 h later and were resuspended in lysis buffer containing 50 mM NaH_2PO_4 (pH 8.0), 300 mM NaCl, 10 mM imidazole, and 1 mg lysozyme. Ruptured by sonication, cell-free extract was prepared by centrifugation at $14,000 \times g$ at 4°C for 45 min. 6His-CsrA was purified by Ni-NTA affinity chromatography (Pierce) according to manufacturer recommendations. The protein-containing fractions were concentrated, and the buffer was exchanged with 10 mM HEPES (pH 7.3), 20 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 10% glycerol. The protein concentration was estimated by the Bradford method, using a Bio-Rad protein assay kit (Bio-Rad).

RNA-EMSA.

Briefly, the CsrA-RNA binding reaction mixtures were prepared using a LightShift chemiluminescent RNA EMSA kit (Thermo Fisher Scientific), following the manufacturer instructions. A total volume of 20 μ l of reaction mixture contained approximately 100 nM 6His-CsrA protein and 2 nM Biotin-labeled RNA and 1 $\times$ binding buffer (10 mM HEPES, pH 7.3, 5% glycerol, 0.1 μ g per microliter of yeast tRNA). After incubation for 20 min at room temperature, 5 μ l of 5 $\times$ loading buffer was added to the mixture. Then, 5 μ l of the resulting mixture was resolved using 6% native polyacrylamide gels in 0.5 $\times$ Tris-borate-EDTA buffer. RNA on the gel was then transferred to a nylon membrane, followed by exposure to 254 nm of UV light for crosslinking. Chemiluminescent signals were visualized by C-DiGit Blot Scanner (LI-COR Biosciences).

Motility, exopolysaccharide production, and biofilm assays.

Bacterial swimming motility was tested using a semisolid NA plate containing 0.3% agar. Bacteria were grown in NB overnight with shaking at 200 rpm using a platform shaker (New Brunswick Scientific) and were then centrifuged down, were washed, and were diluted to $OD_{600} = 0.5$ in sterile water. A 5- μ l suspension of each strain was spotted on the center of the plates and were incubated at 28°C. Plates were photographed after 48 h. The assay was repeated three times independently in triplicates with similar results.

For the motility assay, bacteria were grown overnight in NB medium, and 2 µl of bacterial culture (10^6 CFU/ml) was then plated onto Silva Buddenhagen medium (per liter: 5 g of sucrose, 5 g of yeast extract, 5 g of peptone, and 1 g of glutamic acid, pH 7.0) plus 0.5% (wt/vol) agar (BD Difco). Plates were incubated at 28°C for 48 h. Motility analysis was assessed by examining the circular halo formed by the bacteria. The diameters of the circular halos formed by each strain were measured. The diameter measurements were statistically analyzed using one-way analysis of variance (ANOVA) with Bonferroni ($P < 0.001$) by comparing with the wild-type strain and values were expressed as the means $\pm$ standard deviations of four independent biological replicates.

Formation of biofilms on glass surfaces was examined as described previously, with modifications (Guo et al. 2019; Zhang et al. 2019b). Briefly, the wild-type, mutant, and complemented cells were grown in 2× TY medium (per liter: 16 g of tryptone, 10 g of yeast extract, and 5 g of NaCl) overnight at 28°C and 180 rpm. Bacterial cells were diluted in fresh 2× TY medium to OD₆₀₀ adjusted to 0.1 (UV/Vis Spectrometer Lambda Bio; Perkin Elmer). A volume of 1.5 ml of adjusted bacteria was placed in the glass tubes and was incubated at 28°C without agitation. After 3 days, bacterial growth was checked by measuring the OD₆₀₀, and the culture was removed using a pipette. After repetitive washes with sterile water, the adhered cells were stained with 0.1% (wt/vol) crystal violet (CV) for 30 min at room temperature. After staining by CV, the unbound stain was removed by water washing, and the CV-stained cells were solubilized in 30% acetic acid and were quantified by spectrophotometry at 600 nm. Ratio A (595/600 nm) represents the CV quantification normalized by culture optical density. The means were obtained from three independent biological replicates and standard deviation values were calculated for each strain. Statistical analysis was performed by one-way ANOVA with Bonferroni ($P < 0.001$) by comparing with the wild-type strain.

For exopolysaccharide quantification, bacterial cells were grown overnight at 28°C and 180 rpm in 2× TY medium. The bacteria concentrations were adjusted to 10^8 CFU/mL and 1 mL was transferred to 50 mL of 2× TY plus glucose (2% wt/vol). After 48 h of growth at 28°C and 180 rpm, bacterial cells were pelleted by centrifugation ($2,600 \times g$ for 8 min) and three volumes of 100% ethanol were added to the supernatant. The crude xanthan gum was collected using a glass rod and was placed on a Petri dish to dry at 60°C for 24 h. The assay was performed twice with three biological replicates in each experiment and was statistically analyzed using one-way ANOVA with Bonferroni ($P < 0.001$) by comparing with the wild-type strain.

AUTHOR-RECOMMENDED INTERNET RESOURCE

eggNOG-mapper: <http://eggno-mapper.embl.de/>

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