Prevention of Surface-Associated Calcium Phosphate by the *Pseudomonas syringae* Two-Component System CvsSR

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ABSTRACT CvsSR is a Ca²⁺-induced two-component system (TCS) in the plant pathogen *Pseudomonas syringae* pv. tomato DC3000. Here, we discovered that CvsSR is induced by Fe³⁺, Zn²⁺, and Cd²⁺. However, only supplementation of Ca²⁺ to medium resulted in rugose, opaque colonies in Δ cvsS and Δ cvsR strains. This phenotype corresponded to formation of calcium phosphate precipitation on the surface of Δ cvsS and Δ cvsR colonies. CvsSR regulated swarming motility in *P. syringae* pv. tomato in a Ca²⁺-dependent manner, but swarming behavior was not influenced by Fe³⁺, Zn²⁺, or Cd²⁺. We hypothesized that reduced swarming displayed by Δ cvsS and Δ cvsR strains was due to precipitation of calcium phosphate on the surface of Δ cvsS and Δ cvsR cells grown on agar medium supplemented with Ca²⁺. By reducing the initial pH or adding glucose to the medium, calcium precipitation was inhibited, and swarming was restored to Δ cvsS and Δ cvsR strains, suggesting that calcium precipitation influences swarming ability. Constitutive expression of a CvsSR-regulated carbonic anhydrase and a CvsSR-regulated putative sulfate major facilitator superfamily transporter in Δ cvsS and Δ cvsR strains inhibited formation of calcium precipitates and restored the ability of Δ cvsS and Δ cvsR bacteria to swarm. Lastly, we found that glucose inhibited Ca²⁺-based induction of CvsSR. Hence, CvsSR is a key regulator that controls calcium precipitation on the surface of bacterial cells.

IMPORTANCE Bacteria are capable of precipitating and dissolving minerals. We previously reported the characterization of the two-component system CvsSR in the plant-pathogenic bacterium *Pseudomonas syringae*. CvsSR responds to the presence of calcium and is important for causing disease. Here, we show that CvsSR controls the ability of the bacterium to prevent calcium phosphate precipitation on the surface of cells. We also identified a carbonic anhydrase and transporter that modulate formation of surface-associated calcium precipitates. Furthermore, our results demonstrate that the ability of the bacterium to swarm is controlled by the formation and dissolution of calcium precipitates on the surface of cells. Our study describes new mechanisms for microbially induced mineralization and provides insights into the role of mineral deposits on bacterial physiology. The discoveries may lead to new technological and environmental applications.

KEYWORDS *Pseudomonas syringae*, biomineralization, calcium sensors, carbonic anhydrase, swarming

Two-component systems (TCSs) are mechanisms through which bacteria sense and adapt to environmental changes. They are commonly composed of a transmembrane histidine kinase and a cytoplasmic response regulator (1). Often, upon activation, TCSs regulate genes that are appropriate for the given environment. The genome of the plant-pathogenic bacterium *Pseudomonas syringae* pv. tomato DC3000 encodes 69

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histidine kinases and 95 response regulators (2). Among the TCSs within *P. syringae* pv. tomato DC3000 is a virulence-associated TCS, CvsSR, that is induced by Ca^{2+} (3).

TCSs may be activated or induced by multiple substrates. PhoPQ is activated in *Salmonella enterica* by cationic antimicrobial peptides, Mg^{2+} , Ca^{2+} , and decreases in intracellular pH, and PmrAB in *S. enterica* is induced by both Mg^{2+} and Fe^{3+} (4–6). Transcription of *cvsS* and *cvsR* is upregulated in the presence of Fe(III) citrate (7). CvsSR autoregulates, which suggests that Fe(III) citrate may be an inducer of CvsSR (3). The orthologous TCS to CvsSR in *Pseudomonas aeruginosa*, called BqsSR, is directly activated by Fe^{2+} , and the orthologous TCS in *Haemophilus influenzae*, FirSR, is directly activated by Fe^{2+} and Zn^{2+} (8–10). Given the similarities between CvsSR, BqsSR, and FirSR, metal cations other than Ca^{2+} may induce CvsSR.

CvsSR regulates swarming motility in a Ca^{2+} -dependent manner (3). Both ΔcvsS and ΔcvsR strains are unable to swarm when medium is supplemented with Ca^{2+} . Bacterial swarming motility is negatively impacted by increased exopolysaccharide (EPS) production and decreased flagellar motility (11). CvsSR regulates flagellar biosynthesis genes and alginate biosynthesis genes; however, flagellar motility and overproduction of the EPS alginate were not responsible for inhibiting swarming in the ΔcvsS and ΔcvsR strains (3).

In the presence of extracellular Ca^{2+} , many bacteria precipitate calcium carbonate or calcium phosphate through microbially induced calcium precipitation (MICP) (12). *Pseudomonas* precipitates calcium carbonate or calcium phosphate around colonies or on colonies depending on the strains and growth conditions (13–15). Bacterial cells and EPSs, like alginate, can act as nucleation points for calcium carbonate or calcium phosphate during MICP (16, 17). MICP has been shown to phenotypically change, but not inhibit, swarming motility in *Bacillus* sp. strain JH7 (18).

The opposite of MICP is calcium dissolution or phosphate solubilization in the case of calcium phosphate and apatite (19–21). Phosphate solubilization by bacteria is reported to occur through two possible mechanisms. Through one mechanism, bacteria lower the pH of the external environment through the production of organic acids, such as malic acid, acetic acid, and gluconic acid, in the periplasm, and through a second mechanism, organic anions chelate Ca^{2+} (19, 22). Gluconic acid, in particular, is prominently produced by phosphate-solubilizing bacteria through direct oxidation of glucose by periplasmic glucose dehydrogenase (GCD) (23). Several *Pseudomonas* species perform phosphate solubilization through the secretion of gluconic acid (24, 25). On occasion, inorganic acids, such as carbonic acid, have been implicated in calcium dissolution (21). Carbonic acid is produced by carbonic anhydrases during the hydration of CO_2 and quickly dissociates into bicarbonate and a proton. Carbonic anhydrases in fungi are involved in microbial calcium dissolution (20). In addition, *Bacillus mucilaginosus* and *Brevibacterium linens* BS258 upregulate transcription of genes that code for carbonic anhydrases during calcium dissolution (26, 27). CvsSR directly regulates expression of the beta-carbonic anhydrase, PSPTO_5255 (here, 5255), and a major facilitator superfamily (MFS) transporter, PSPTO_5256 (here, 5256), that are found within the same putative operon (3). MFS transporters found in the same operon with a carbonic anhydrase could potentially function as bicarbonate or carbonic acid transporters (28).

In this paper, we show that *cvsS* and *cvsR* are induced by several metal cations other than Ca^{2+} but that swarming motility is impacted only by supplementation with Ca^{2+} . We then show that surface-associated MICP occurs in ΔcvsS and ΔcvsR strains when supplemental Ca^{2+} is added to medium and, furthermore, that MICP is likely involved in disrupting swarming motility. Last, we show that the carbonic anhydrase PSPTO_5255 and the MFS transporter PSPTO_5256 are involved in inhibition of surface-associated MICP in *P. syringae* pv. tomato DC3000.

RESULTS

***cvsS* and *cvsR* expression levels are induced by multiple metal cations.** We reported previously that transcription of *cvsSR* was elevated in medium with Fe(III)

citrate added compared to levels in iron-free medium (7, 9). Using a luciferase reporter gene construct for the promoter of *cvsSR* (P_{cvsSR}), we previously showed that *cvsSR* was induced by Ca^{2+} and autoregulates (3). Ca^{2+} -based induction of *cvsSR* could also be inhibited with the addition of the Ca-specific chelator EGTA (3). To confirm that expression of *cvsSR* is influenced by Fe^{3+} and to test if other metal cations induce expression of *cvsSR*, we used our reporter system. We found that Fe^{3+} , Zn^{2+} , and Cd^{2+} induced transcription of *cvsSR* (Fig. 1A to C). Thus, *cvsSR* can be induced by multiple metal cations.

Other metal cations do not inhibit swarming motility of $\Delta cvsS$ and $\Delta cvsR$ strains. One of the prominent phenotypes $\Delta cvsS$ and $\Delta cvsR$ strains displayed was reduced swarming on nutrient broth (NB) swarming agar supplemented with Ca^{2+} (3). To determine whether addition of Fe^{3+} , Zn^{2+} , and Cd^{2+} also inhibited swarming in $\Delta cvsS$ and $\Delta cvsR$ strains, we tested swarming motility of the $\Delta cvsS$ and $\Delta cvsR$ strains compared to that of the wild type (WT) on NB swarming agar supplemented with Fe^{3+} , Zn^{2+} , or Cd^{2+} . We found that levels of swarming motility were similar between WT, $\Delta cvsS$, and $\Delta cvsR$ strains under these conditions (see Fig. S1 in the supplemental material). These data support the idea that inhibition of swarming motility is associated with the addition of Ca^{2+} .

$\Delta cvsS$ and $\Delta cvsR$ strains are rugose and opaque on calcium-supplemented medium. In previous swarming assays performed on NB medium supplemented with Ca^{2+} , $\Delta cvsS$ and $\Delta cvsR$ colonies were opaquer than the WT (3). This opaque phenotype was also visible on $\Delta cvsS$ and $\Delta cvsR$ colonies when they were grown on NB agar supplemented with Ca^{2+} (Fig. 2A). In comparison, the WT and a $\Delta cvsR$ complemented strain, created using an insertion in *trans* of the native promoter for *cvsSR* and the genes PSPTO_3383, PSPTO_3382, and *cvsR* ($\Delta cvsRc$ strain), formed clear, smooth colonies (Fig. 2A). The phenotype of $\Delta cvsS$ and $\Delta cvsR$ strains was Ca^{2+} specific since opaque colonies did not form on NB medium or NB medium supplemented with $Fe(III)$ citrate, Zn^{2+} , or Cd^{2+} (Fig. S2). The opaque nature of $\Delta cvsS$ and $\Delta cvsR$ colonies when they were grown on NB agar supplemented with Ca^{2+} was similar in appearance to that of *Pseudomonas* strains that precipitated calcium phosphate on the surface of colonies when they were grown under the same conditions (15). To test for calcium precipitate, we stained WT, $\Delta cvsS$, $\Delta cvsR$, and $\Delta cvsRc$ strains grown on NB agar supplemented with Ca^{2+} or NB swarming agar supplemented with Ca^{2+} with alizarin red S (ARS) (Fig. 2A and Fig. S3). ARS is a dye that stains calcium-rich areas and has been shown to stain areas of calcium precipitate on or around *Pseudomonas* colonies (15). $\Delta cvsS$ and $\Delta cvsR$ cells stained with ARS within a day of spotting, while WT and $\Delta cvsRc$ cells were not stained. This suggests that $\Delta cvsS$ and $\Delta cvsR$ strains concentrate calcium on the surface of colonies when supplemental Ca^{2+} is added to the medium.

$\Delta cvsS$ and $\Delta cvsR$ strains precipitate amorphous calcium phosphate on the cell surface. ARS stains calcium-rich areas, which could represent calcium precipitates or areas of abundant chelated Ca^{2+} . To determine whether the surface of $\Delta cvsS$ and $\Delta cvsR$ colonies contained calcium precipitates, the colonies were analyzed using both X-ray diffraction (XRD) and *in vivo* Raman spectroscopy. XRD of dried WT, $\Delta cvsS$, and $\Delta cvsR$ colonies grown on NB agar supplemented with Ca^{2+} showed amorphous apatite present on the $\Delta cvsS$ and $\Delta cvsR$ colonies but not on the WT colony (Fig. 2B). *In vivo* Raman spectroscopy from colonies after 1 day of growth on NB agar supplemented with Ca^{2+} showed that the surface of live $\Delta cvsS$ and $\Delta cvsR$ colonies produced amorphous calcium phosphate, in contrast to growth of WT colonies (Fig. 2C). Therefore, these data suggest that *CvsSR* directly or indirectly regulated genes involved in preventing calcium phosphate precipitation on the surface of cells.

Swarming inability in $\Delta cvsS$ and $\Delta cvsR$ strains is pH dependent. Decreased swarming and surface-associated MICP on $\Delta cvsS$ and $\Delta cvsR$ strains occurred only on NB medium supplemented with Ca^{2+} . This suggested a connection between swarming ability and MICP. Mixing Ca^{2+} and phosphate *in vitro* can spontaneously precipitate calcium phosphate; however, the rate of precipitate formation is dependent on pH (29).

FIG 1 Luminescence assays to assess activity of P_{cvsSR} in WT grown in MG medium compared to growth in MG medium supplemented with 50 μ M Fe(III) citrate (A), MG medium supplemented with 50 μ M ZnCl₂ (B), or MG medium supplemented with 10 μ M CdCl₂ (C). The relative luminescence was calculated by using the total luminescence relative to the OD₆₀₀ value. Experiments were performed three times. The three experiments were compiled using least squares mean (LSM) regression. The error bars represent standard deviations generated by the differences observed between samples. Statistically significant differences were determined using a Tukey honestly significant differences test (*, $P < 0.01$).

Using the pH indicator dye bromothymol blue (BB), we found that the WT, $\Delta cvsS$, and $\Delta cvsR$ strains all raised the pH of surrounding medium over time (Fig. S4). Changes in pH over time may influence the rate at which MICP occurs. We thought that lowering the initial pH of NB agar supplemented with Ca²⁺ may delay or reduce calcium precipitation and, thus, influence swarming ability. The WT, $\Delta cvsS$, $\Delta cvsR$, and $\Delta cvsRc$ strains were spotted on NB swarming agar plates at pHs of 6.7, 6.2, 5.8, and 5.3 with or

FIG 2 (A) The WT, $\Delta cvtS$, $\Delta cvtR$, and $\Delta cvtRc$ strains grown on NB agar supplemented with 5 mM $CaCl_2$ after 1 day of growth with (+) and without (–) alizarin red S (ARS) staining. These pictures are representative of experiments that were repeated three times. (B) X-ray diffraction patterns from WT, $\Delta cvtS$, and $\Delta cvtR$ cultures grown on NB medium supplemented with 5 mM $CaCl_2$ for 1 day. Values for the 2θ angle from 20° to 50° are shown. Peaks in the spectra for the $\Delta cvtS$ and $\Delta cvtR$ strains are for amorphous apatite. (C) Raman spectroscopy patterns from WT, $\Delta cvtS$, and $\Delta cvtR$ cultures grown on NB medium supplemented with 5 mM $CaCl_2$ for 1 day. The peak centered at 955 cm^{-1} is expected when amorphous calcium phosphate is present. This peak is indicated in the $\Delta cvtS$ and $\Delta cvtR$ strain spectra with a blue dot.

without supplemental Ca^{2+} . WT, $\Delta cvtS$, $\Delta cvtR$, and $\Delta cvtRc$ strains swarmed more on NB agar plates without additional Ca^{2+} at pH values of 6.2, 5.8, and 5.3 compared to swarming at a pH of 6.7 (Fig. S5). Similarly, the WT swarmed more on NB swarming agar plates supplemented with Ca^{2+} at pH values of 6.2, 5.8, and 5.3 than at a pH of 6.7 (Fig. 3 and Fig. S6). A similar trend occurred in the $\Delta cvtRc$ strain as well; however, the swarming diameter of $\Delta cvtRc$ colonies was on average reduced compared to that of the WT and was not as large as diameters of the $\Delta cvtS$ and $\Delta cvtR$ strains at a pH of 5.3 (Fig. 3 and Fig. S6). In comparison to levels for the WT and $\Delta cvtRc$ strains, swarming in the $\Delta cvtS$ and $\Delta cvtR$ strains began to increase at a pH of 5.8 on NB medium supplemented with Ca^{2+} and was fully recovered to WT levels at a pH of 5.3 (Fig. 3 and Fig. S6). The recovery of swarming in $\Delta cvtS$ and $\Delta cvtR$ strains at a lower pH on NB swarming agar supplemented with Ca^{2+} suggests that a pH-labile product produced by $\Delta cvtS$ and $\Delta cvtR$ strains may be involved in inhibiting swarming motility. Given that $\Delta cvtS$ and $\Delta cvtR$ strains precipitated calcium phosphate on the surface of cells when they were

FIG 3 Diameters of WT, $\Delta cvsS$, $\Delta cvsR$, and $\Delta cvsRc$ colonies when grown on NB swarming agar supplemented with 5 mM $CaCl_2$ at the indicated pH values measured after 18 to 20 h of growth. Swarming assays were performed three independent times. Recorded measurements from each experiment were compiled and averaged. The data shown are averaged from three technical replicates from a single experiment. Similar trends were observed between experiments. Significant differences between the measured diameters of the $\Delta cvsS$ and $\Delta cvsR$ strains and the WT diameter according to one-way analysis of variance are indicated (*, $P < 0.01$).

grown on NB agar supplemented with Ca^{2+} and that spontaneous calcium phosphate precipitation *in vitro* can be inhibited at lower pH, it is possible that the decreased swarming observed in the $\Delta cvsS$ and $\Delta cvsR$ strains on NB medium supplemented with Ca^{2+} is linked to surface-associated MICP.

Calcium phosphate precipitation in $\Delta cvsS$ and $\Delta cvsR$ strains is inhibited by the addition of glucose to medium. Calcium phosphate can be solubilized by bacteria by lowering the pH through the secretion of gluconic acid during a process called phosphate solubilization (30). Oxidation of glucose to gluconic acid by bacteria requires glucose dehydrogenase (GDH) and the cofactor pyrroloquinoline quinone (PQQ). The genome of *P. syringae* pv. tomato DC3000 includes a gene that encodes glucose dehydrogenase (*gdh*) and a gene cluster that can be utilized to produce PQQ (31). Therefore, *P. syringae* pv. tomato DC3000 should be able to oxidize glucose to gluconic acid and lower environmental pH when glucose is present as a carbon source. The WT, $\Delta cvsS$, and $\Delta cvsR$ strains produced molar concentrations of gluconic acid when grown in NB medium supplemented with Ca^{2+} and glucose (Table 1). In comparison, only millimolar concentrations of gluconic acid were produced by the WT, $\Delta cvsS$, and $\Delta cvsR$ strains when grown in NB medium, NB medium supplemented with glucose, NB medium supplemented with Ca^{2+} , or NB medium supplemented with Ca^{2+} and the glucose analog methyl- α -glucopyranoside (MAP) (Table 1). This suggests that Ca^{2+} , together with glucose, stimulates gluconic acid production and that CvsSR is not required for gluconic acid production. As expected, the WT, $\Delta cvsS$, and $\Delta cvsR$ strains lowered the pH of the environment when grown on NB agar plates supplemented with

TABLE 1 Concentration of gluconate/gluconic acid in *P. syringae* pv. tomato DC3000 spent medium

Condition	Mean (SD) gluconate concn (mM) in ^a :		
	WT	$\Delta cv sS$ strain	$\Delta cv sR$ strain
NB	2.2 (1.4)	12.8 (1.0)	16.5 (4.2)
NB + CaCl ₂	4.4 (2.2)	14.3 (7.1)	7.6 (1.5)
NB + glucose	66.6 (7.9)	87.5 (86.7)	130.2 (75.8)
NB + CaCl ₂ + glucose	1,672.8 (87.4)	2,190.9 (22.5)	2,001.8 (85.0)
NB + CaCl ₂ + MAP	7.2 (4.5)	9.7 (2.9)	7.4 (0.7)

^aThe mean concentration of gluconate/gluconic acid was determined by averaging three independent biological replicates, and the standard deviation (SD) was determined.

Ca²⁺ and glucose and raised the pH of the environment when grown on NB agar plates supplemented with Ca²⁺ and the glucose analog MAP (Fig. S7). When the WT, $\Delta cv sS$, $\Delta cv sR$, and $\Delta cv sRc$ strains were grown on NB agar supplemented with Ca²⁺ and glucose, the $\Delta cv sS$ and $\Delta cv sR$ colonies looked similar to those of the WT and were not stained by ARS (Fig. 4). In comparison, $\Delta cv sS$ and $\Delta cv sR$ strains grown on NB agar supplemented with Ca²⁺ and MAP were rugose and opaque and were stained by ARS (Fig. 4). This suggests that surface-associated MICP did not occur on $\Delta cv sS$ and $\Delta cv sR$ colonies when they were grown on NB medium supplemented with Ca²⁺ and glucose (Fig. 4), and the lack of MICP under these conditions is likely due to the secretion of gluconic acid by these strains.

To further establish that calcium phosphate precipitation is linked to decreased swarming, we added glucose or MAP to NB swarming agar supplemented with Ca²⁺ and tested swarming of the WT, $\Delta cv sS$, $\Delta cv sR$, and $\Delta cv sRc$ strains (Fig. 5). Swarming of the $\Delta cv sS$ and $\Delta cv sR$ strains was similar to that of the WT when glucose was added to NB swarming agar supplemented with Ca²⁺ (Fig. 5A and B). Similar to what was seen on NB swarming agar, the $\Delta cv sRc$ strain appeared to swarm less than the other strains (Fig. 5A and B and S5). In comparison, when MAP was added to NB swarming agar supplemented with Ca²⁺, swarming remained inhibited in the $\Delta cv sS$ and $\Delta cv sR$ strains, and the strains precipitated calcium phosphate on the surface of cells (Fig. 5C and D). The $\Delta cv sRc$ strain was able to swarm more than the $\Delta cv sR$ strain on the NB swarming agar supplemented with Ca²⁺ and MAP (Fig. 5C and D). These results suggest that MICP on $\Delta cv sS$ and $\Delta cv sR$ strains is involved in reducing swarming motility.

FIG 4 WT, $\Delta cv sS$, $\Delta cv sR$, and $\Delta cv sRc$ strains grown on NB agar supplemented with 5 mM CaCl₂ and 0.5% (wt/vol) glucose (A) or NB agar supplemented with 5 mM CaCl₂ and 0.5% (wt/vol) MAP (B) after 1 day of growth with (+) and without (–) ARS staining. These pictures are representative of experiments that were repeated three times.

FIG 5 Photos and measured diameters of WT, ΔcvsS , ΔcvsR , and ΔcvsRc strains swarming on NB medium supplemented with CaCl_2 and glucose (A and B) or on NB medium supplemented with CaCl_2 and MAP (C and D). Diameters of the strains were measured after 18 to 20 h of growth on swarming plates. Error bars are representative of standard errors between three separate experiments. Significant differences in diameters between the WT and the given strain (*) and between the diameters of the ΔcvsR and ΔcvsRc strains (**) were determined using Student's two-tailed *t* test ($P < 0.01$). These experiments were repeated three times, and the values of the measured diameters were averaged.

CvsSR regulates a carbonic anhydrase and putative sulfate transporter involved in calcium dissolution in *P. syringae* pv. tomato DC3000. We searched the regulon of CvsSR for possible gene candidates that might play a role in calcium dissolution or phosphate solubilization. Microbes can dissolve calcium minerals through the use of carbonic anhydrases (20). CvsSR regulates the beta-carbonic anhydrase PSPTO_5255 (5255) found in a putative operon with a putative sulfate permease PSPTO_5256 (5256) (3). We performed overlapping reverse transcription-PCR (RT-PCR) and found that 5255 and 5256 were transcribed together within the same operon (Fig. S8). To test whether the carbonic anhydrase or the permease could prevent the formation of surface-associated calcium precipitation in *P. syringae* pv. tomato DC3000, we grew the WT, $\Delta 5255$, $\Delta 5256$, and $\Delta 5255/5256$ strains and a $\Delta 5255$ complement that constitutively expressed 5255 ($\Delta 5255c$) and a $\Delta 5256$ complement that constitutively expressed 5256 ($\Delta 5256c$) on NB agar supplemented with Ca^{2+} . ARS stained the surface

FIG 6 Photos of WT, $\Delta 5255$, $\Delta 5256$, $\Delta 5255/5256$, $\Delta 5255c$, and $\Delta 5256c$ strains after 1, 2, and 3 days of growth on NB medium supplemented with Ca^{2+} before and after ARS staining. A zone of clearing/brown halo can be seen after 2 days of growth around the $\Delta 5255c$ and $\Delta 5256c$ strains while a white halo is still surrounding the WT, $\Delta 5255$, $\Delta 5256$, and $\Delta 5255/5256$ strains. After 3 days of growth, only the $\Delta 5255$ strain still had a white halo present. This experiment was independently repeated three times, and these pictures are representative of those experiments. (B) Raman spectra of WT, $\Delta 5255$, $\Delta 5256$, and $\Delta 5255/5256$ strains grown on NB medium supplemented with Ca^{2+} . Peaks normally associated with biological organisms can be seen in the spectra. A peak for apatite is expected at 960 cm^{-1} and is not present in the $\Delta 5255$, $\Delta 5256$, and $\Delta 5255/5256$ strain spectra.

of the $\Delta 5255$ and $\Delta 5255/5256$ strains after 1 day of growth but did not stain the $\Delta 5256$ strain, suggesting that the centers of the $\Delta 5255$ and $\Delta 5255/5256$ colonies were Ca^{2+} rich (Fig. 6A). However, *in vivo* Raman spectroscopy did not detect any calcium phosphate on the surface of the $\Delta 5255$ and $\Delta 5255/5256$ strains (Fig. 6B). One interesting phenotype of the $\Delta 5255c$ and $\Delta 5256c$ strains was that they had a prominent zone of clearing between the colony and the white halo around the colonies or a brown halo after 2 days of growth, while the WT, $\Delta 5255$, $\Delta 5256$, and $\Delta 5255/5256$ strains displayed only a white halo (Fig. 6A). By the third day of growth, the WT and $\Delta 5256$ had formed a brown halo while the $\Delta 5255$ and $\Delta 5255/5256$ strains still produced only a white halo around the colonies. The white halo has previously been shown to be composed of amorphous calcium phosphate that later is replaced by a brown halo of amorphous apatite (15). This zone of clearing and the subsequent formation of amorphous apatite could possibly be due to calcium dissolution by *P. syringae* pv. tomato DC3000. The early appearance of the brown halo in the $\Delta 5255c$ and $\Delta 5256c$ strains before that in the WT suggests that 5255 and 5256 may play a role in calcium dissolution and that constitutive expression of these genes deregulates the process of calcium precipitation and calcium dissolution by *P. syringae* pv. tomato DC3000.

Expression of 5255 and 5256 prevents calcium precipitation on the surface of $\Delta cvsS$ and $\Delta cvsR$ cells. We hypothesized that constitutive expression of 5255 or 5256 in $\Delta cvsS$ or $\Delta cvsR$ cells may prevent calcium phosphate from accumulating on the

surface of colonies. To determine if 5255 and 5256 were involved in calcium dissolution, the Δ cvsS strain constitutively expressing 5255, 5256, or 5255 and 5256 (Δ cvsS pBS46::5255, Δ cvsS pBS46::5256, and Δ cvsS pBS46::5255/5256 strains, respectively) and the Δ cvsR strain constitutively expressing 5255, 5256, or 5255 and 5256 (Δ cvsR pBS46::5255, Δ cvsR pBS46::5256, and Δ cvsR pBS46::5255/5256 strains, respectively) were spotted on NB agar supplemented with Ca^{2+} and observed for 3 days. Constitutive expression of 5255 or 5256 reduced calcium precipitation on the Δ cvsS strain after 1 or 2 days of spotting but did not affect calcium precipitation by the Δ cvsR strain (Fig. 7A and B). However, constitutive expression of both 5255 and 5256 in the Δ cvsS and Δ cvsR strains prevented calcium phosphate precipitation on the colonies and restored the WT phenotype (Fig. 7A and B). Raman spectroscopy of these strains after 1 day showed that the Δ cvsS pBS46::5255, Δ cvsR pBS46::5255, and Δ cvsR pBS46::5256 strains precipitated calcium phosphate on the surface of cells and that the Δ cvsS pBS46::5256, Δ cvsS pBS46::5255/5256, and Δ cvsR pBS46::5255/5256 strains did not precipitate calcium phosphate on the surface of cells (Fig. 7C and D). These data suggest that 5255 and 5256 are able to prevent calcium phosphate precipitation on the surface of *P. syringae* pv. tomato DC3000 cells.

Since previous experiments suggested that calcium precipitation impeded swarming motility, we performed swarming assays on Δ cvsS and Δ cvsR cells constitutively expressing 5255, 5256, or 5255 and 5256 and compared the levels of motility of these strains to those of the Δ cvsS and Δ cvsR strains. Only the Δ cvsS pBS46::5256, Δ cvsS pBS46::5255/5256, and Δ cvsR pBS46::5255/5256 strains swarmed significantly more than the Δ cvsS or Δ cvsR strains when they were grown on NB medium supplemented with Ca^{2+} (Fig. 8). None of these three strains precipitated any detectable calcium phosphate on the surface of cells after 1 day of growth on NB medium supplemented with Ca^{2+} . In comparison, the Δ cvsS pBS46::5255, Δ cvsR pBS46::5255, and Δ cvsR pBS46::5256 strains, which still precipitated calcium phosphate on the surface of cells after 1 day, did not swarm (Fig. 8). These data suggest that calcium precipitation can impede swarming in *P. syringae* pv. tomato DC3000 and is at least partially involved in inhibiting swarming in Δ cvsS and Δ cvsR strains when they are grown on NB medium supplemented with Ca^{2+} .

Glucose represses Ca^{2+} -based induction of CvsSR. We found that *P. syringae* pv. tomato DC3000 can secrete gluconic acid or use CvsSR-regulated genes to prevent surface-associated calcium phosphate precipitation. We wanted to determine whether expression levels of *cvsSR* and 5255 were influenced in medium supplemented with Ca^{2+} and glucose. Using *P. syringae* pv. tomato DC3000 with the reporter gene constructs with P_{cvsSR} or the promoter for 5255/5256 (P_{5255}), we determined that the addition of glucose to medium supplemented with Ca^{2+} repressed induction of *cvsSR* and 5255/5256 (Fig. 9). We believe that these data support a model whereby *P. syringae* pv. tomato DC3000 can utilize two distinct pathways for preventing MICP on the surface of cells when the environment is replete with Ca^{2+} . One pathway is through the secretion of gluconic acid if glucose is present, and the other is through the activation of CvsSR and regulation of genes within the CvsSR regulon, including 5255 and 5256 (Fig. 10).

DISCUSSION

Here, we show that, in addition to Ca^{2+} , Fe^{3+} , Zn^{2+} , and Cd^{2+} induce expression of *cvsSR* (3). However, the Δ cvsS and Δ cvsR strains showed only a noticeable phenotype of surface-associated MICP when grown on medium supplemented with Ca^{2+} . This surface-associated MICP on Δ cvsS and Δ cvsR cells had consequences for the bacteria. Notably, we found that surface-associated MICP in Δ cvsS and Δ cvsR strains dramatically reduced swarming motility on medium supplemented with Ca^{2+} . Decreased swarming motility in Δ cvsS and Δ cvsR strains was recovered by reducing the initial pH of the swarming agar, producing conditions that enable *P. syringae* pv. tomato DC3000 to secrete gluconic acid (by addition of glucose to the medium), or by constitutively

FIG 7 (A and B) Photos of the indicated strains after 1, 2, and 3 days of growth on NB medium supplemented with Ca^{2+} before (–) and after (+) ARS staining. ARS stained the $\Delta cvaS$, $\Delta cvaS$ pBS46:5255, $\Delta cvaS$ pBS46:5256, $\Delta cvrR$, $\Delta cvrR$ pBS46:5255, and $\Delta cvrR$ pBS46:5256 strains all 3 days but did not stain the $\Delta cvaS$ pBS46:5255/5256 and $\Delta cvrR$ pBS46:5255/5256 strains. These photos are representative of three independent experiments. (C and D) Raman spectra of the indicated strains grown on NB medium supplemented with Ca^{2+} after 1 day of growth. Peaks normally associated with biological organisms can be seen in the spectra. A peak for apatite is expected at 960 cm^{-1} , and a slightly downshifted peak centered around 955 cm^{-1} can be seen in spectra of some strains, as indicated (blue dot).

expressing the CvsSR-regulated genes 5255 and 5256. In the last two cases, both strategies also prevented MICP on the surface of $\Delta cvaS$ and $\Delta cvrR$ cells.

TCSs can be induced by multiple signals. PhoPQ responds to cationic antimicrobial peptides, Mg^{2+} , Ca^{2+} , and acidic conditions (4). ColSR responds to Fe^{3+} , Zn^{2+} , Mn^{2+} , and Cd^{2+} (32). Thus, the fact that expression of *cvsSR* is induced by Fe^{3+} , Zn^{2+} , Cd^{2+} , and Ca^{2+} is not unusual (3). Ca, Zn, and Fe are known to be present in the plant

FIG 8 Photos and measured diameters of the indicated strains grown on NB swarming agar supplemented with Ca^{2+} . These photos are representative of swarming assays performed three separate times. Significant differences in swarming diameters between those of the indicated strains and the ΔcvsS strain or the ΔcvsR strain were determined using one-way analysis of variance (*, $P < 0.01$). These data are the average of three technical replicates from a single experiment and are representative of what was observed when the experiment was performed three independent times.

apoplast (3). It is possible that the presence of all three metals within the apoplast acts cumulatively to induce expression of *cvsSR*. The presence of Fe and Zn in the apoplast could explain why the addition of the Ca^{2+} -specific chelator EGTA to apoplastic washing fluid only partially reduced expression of *cvsSR* (3). Identification of a synergistic behavior between Zn^{2+} , Fe^{3+} , Cd^{2+} , and Ca^{2+} in inducing transcription of *cvsSR* would be logical to investigate in future studies.

The mechanism behind MICP, like that seen on ΔcvsS and ΔcvsR cells, is believed to be a passive process that occurs due to the nucleation of Ca^{2+} on or around bacterial cells. Negatively charged exopolysaccharides, like alginate, are thought to be associated with MICP (16, 33). ΔcvsS and ΔcvsR *P. syringae* strains produce more alginate than the WT; however, we found that surface-associated calcium precipitation occurred even on ΔcvsS ΔalgD and ΔcvsR ΔalgD strains (see Fig. S9 in the supplemental material) (20). When cells are grown on NB agar supplemented with Ca^{2+} , calcium phosphate will normally precipitate in the region surrounding *P. syringae* pv. tomato DC3000 colonies but not on the surface of colonies (15). The fact that deletion of *CvsSR* results in surface-associated MICP suggests that *P. syringae* pv. tomato DC3000 utilizes *CvsSR* to actively prevent calcium phosphate from accumulating on the surface of cells. Thus, it may be appropriate to say that surface-associated MICP on ΔcvsS and ΔcvsR colonies represents actually a loss of calcium phosphate dissolution by *P. syringae* pv. tomato DC3000 under certain conditions.

It is still unclear what factors bind calcium and phosphate and promote calcium phosphate precipitation on the ΔcvsS and ΔcvsR strains. A previous transposon mutant screen of *P. syringae* pv. tomato DC3000 found that the TetR-like transcription factor TvrR is necessary to prevent MICP on the surface of bacterial cells (15). TetR-like transcription factors commonly repress the transcription of genes, and TvrR may repress transcription of a gene that codes for a Ca^{2+} -binding protein involved in calcium precipitation (34). Likewise, it is possible that a gene repressed by *CvsR* codes for a Ca^{2+} -binding protein that coordinates MICP on the surface of cells. There are known examples of Ca^{2+} -binding proteins that facilitate the precipitation of apatite. A proteolipid from *Corynebacterium matruchotii* coordinates Ca^{2+} and PO_4 in conjunction with phospholipids to precipitate apatite on the surface of bacterial cells (35). Specific

FIG 9 Luminescence assays to assess activity of P_{cvSR} (A) or P_{5255} (B) when strains were grown in MG medium supplemented with Ca^{2+} or MG medium supplemented with Ca^{2+} and glucose. The relative luminescence was determined by normalizing the total luminescence to the OD_{600} . Three independent experiments were compiled using least squares mean (LSM) regression. The error bars represent standard errors between the three experiments. Significant differences in relative luminescence values between results at a given time point with MG medium supplemented with Ca^{2+} and MG medium supplemented with Ca^{2+} and glucose were determined using a Tukey honestly significant differences test (*, $P < 0.01$).

proteins in animals, such as dentin, coordinate Ca^{2+} and PO_4 to precipitate apatite (36). Many genes that code for uncharacterized proteins were upregulated in the Δcvr strain compared to the WT level when bacteria were grown on NB medium supplemented with Ca^{2+} , and one of these uncharacterized proteins could be a Ca^{2+} -binding protein that coordinates calcium phosphate precipitation (3). Further investigation into the genes involved in MICP and calcium dissolution in *P. syringae* pv. tomato DC3000 could lead to the identification of proteins that bind Ca^{2+} and cause calcium phosphate precipitation on the surface of cells.

The $\Delta cvrS$ and $\Delta cvrR$ strains have not lost the ability to secrete gluconic acid and, like the WT, can prevent the build-up of MICP on the surface of cells if glucose is present in the medium. Much as in the case of *P. syringae* pv. tomato, the addition of Ca^{2+} to medium stimulates gluconic acid production in *P. aeruginosa*, *Escherichia coli*, and *Klebsiella pneumoniae* when glucose is available as a carbon source (37–40). The $CvsSR$ regulon in *P. syringae* pv. tomato includes over 200 genes, and under the conditions we tested, *gcd* is not part of the $CvsSR$ regulon (3). Given that *cvsSR* expression is repressed by the addition of glucose, it appears that $CvsSR$ and the genes in the $CvsSR$ regulon are utilized by *P. syringae* pv. tomato DC3000 only when glucose is not present as a method to prevent MICP on the surface of cells. Therefore, *P. syringae* pv. tomato DC3000 has at least two distinct pathways for calcium dissolution.

We found that 5255 and 5256 are two of the genes that $CvsSR$ regulates to prevent surface-associated MICP. The annotated roles of 5255 and 5256 are those of a carbonic

FIG 10 Model depicting signaling involved in *P. syringae* pv. tomato-mediated prevention of calcium phosphate precipitation through a CvsSR-mediated pathway when glucose is not present or through glucose dehydrogenase (GCD) and PQQ when glucose is present.

anhydrase and a putative SulP MFS transporter, respectively. Carbonic anhydrases hydrate CO_2 to H^+ and HCO_3^- , and these products are capable of dissolving limestone (20). SulP MFS transporters that are found in an operon with a carbonic anhydrase may transport bicarbonate (28). 5255 and 5256 may be used as a way for *P. syringae* pv. tomato DC3000 to secrete bicarbonate or carbonic acid and solubilize or prevent the accumulation of calcium phosphate on the surface of cells. Bacterial use of carbonic anhydrases for calcium dissolution may be common. Both *B. mucilaginosus* and *B. linens* BS258 upregulate expression of carbonic anhydrases during calcium dissolution (26, 27). Further study of 5255, 5256, or bacterial calcium dissolution could determine whether there is a shared mechanism behind bacterial calcium dissolution that utilizes carbonic anhydrases and transporters.

Identifying a causal factor behind the reduced swarming phenotype observed in the ΔcvsS and ΔcvsR strains when Ca^{2+} was added to medium was one of the original driving factors behind this study. Swarming was recovered in the ΔcvsS and ΔcvsR strains when the pH of medium was lowered. Calcium phosphate precipitation is pH labile, and these results coupled with the fact that swarming was completely or partially recovered when the ΔcvsS or ΔcvsR strains did not precipitate calcium phosphate on the surface of cells strongly suggest that surface-associated calcium phosphate precipitation was the causal factor behind inhibited swarming in these strains. Swarming motility in bacteria is dependent on bacteria reducing the surface tension of the liquid around them. It is for this reason that bacteria produce biosurfactants that lower the surface tension of water during swarming motility (41). Calcium precipitation on the surface of cells may inhibit swarming by creating a physical barrier that prevents motility or influence the multicellular behavior of the cells and promote bacterial cell-cell interactions.

Although the ΔcvsRc strain complemented swarming of the ΔcvsS and ΔcvsR strains on NB medium supplemented with Ca^{2+} , it also showed reduced swarming compared to that of the WT in most of the swarming assays. The reduced swarming in this strain was also less than that of the ΔcvsS and ΔcvsR strains when they were grown on NB

swarming agar supplemented with Ca^{2+} that was at an initial pH of 5.3 (Fig. 3D). The ΔcvsRc strain has a second copy of PSPTO_3383 and PSPTO_3382 compared to the sequence of the WT. Both of these genes code for putative membrane-bound proteins with PepSY inhibitory domains (31). It is possible that the second copies of PSPTO_3383 and PSPTO_3382 cause secondary effects that affect swarming in *P. syringae* pv. tomato DC3000.

Reduced swarming on medium supplemented with Ca^{2+} was not observed in deletion mutants for the orthologous TCS to CvsSR in *P. aeruginosa* PAO1 (42). However, those swarming assays were performed on BM2 medium, which uses glucose as a carbon source. Since the genome of *P. aeruginosa* PAO1 expresses *gcd* and the PQQ biosynthetic gene cluster, it is possible that BM2 medium supplemented with Ca^{2+} promotes the production of gluconic acid in *P. aeruginosa* in much the same way that NB medium supplemented with glucose and Ca^{2+} does in *P. syringae* pv. tomato DC3000 (43). If this is the case, it could explain the differing swarming results between the ΔcvsS and ΔcvsR *P. syringae* pv. tomato strains and the ΔcarS and ΔcarR *P. aeruginosa* PAO1 strains.

Ca^{2+} is abundant in the leaf apoplast, with concentrations around 10 mM (3, 44). This concentration is not static during biotic stress. Studies in *Phaseolus vulgaris* infected with *P. syringae* pv. phaseolicola RJ3 suggest that apoplastic Ca^{2+} concentrations actually increase during a compatible interaction (44). Given the high concentration of Ca^{2+} in the apoplast, it is possible that *P. syringae* precipitates calcium at some point during growth *in planta* and could then dissolve calcium precipitates. Ca^{2+} is an important signaling molecule during pattern-triggered immunity (PTI) (45). Some bacterial plant pathogens, but not *P. syringae* pv. tomato, require EPSs to chelate Ca^{2+} as a way to reduce PTI (46). Given that CvsSR is involved in *P. syringae* pv. tomato DC3000 virulence, MICP and calcium dissolution may be employed by *P. syringae* pv. tomato DC3000 during growth *in planta* as a way to reduce or disable PTI (3). Follow-up studies on the role of CvsSR during *P. syringae* pv. tomato DC3000 growth *in planta* could help identify whether *P. syringae* pv. tomato precipitates and/or dissolves calcium during growth *in planta*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and primers used in this study can be found in Tables S1 and S2, respectively, in the supplemental material. *P. syringae* pv. tomato was routinely cultured on King's B (KB) agar (47). Experiments were performed in nutrient broth (NB) medium (Becton, Dickinson, Franklin Lakes, NJ) or mannitol glutamate (MG) medium (7). *E. coli* was routinely cultured on lysogeny broth medium.

Motility assays. Swarming assays were performed using 0.5% agar NB plates that were measured to be at a pH of 6.7, 6.2, 5.8, or 5.3, with the following reagents added when appropriate: 5 mM CaCl_2 , 50 μM Fe(III) citrate, 50 μM ZnCl_2 , 10 μM CdCl_2 , 0.5% (wt/vol) D-glucose, or 0.5% (wt/vol) MAP (Sigma-Aldrich). *P. syringae* pv. tomato strains were grown overnight in KB medium to stationary phase, and 5 μl of each culture was spotted on a swarming plate. Swarming zones were measured after plates were incubated for 18 to 20 h at room temperature. Student's two-tailed *t* test was used to determine statistical significance. Photos of swarming plates were taken after plates were incubated for 18 to 20 h at room temperature.

Calcium precipitation assays. *P. syringae* pv. tomato strains were grown overnight in KB medium, washed twice in NB medium, and then resuspended in NB medium at an optical density at 600 nm (OD_{600}) of 0.3. Five microliters of each culture was spotted onto NB agar plates supplemented with 5 mM CaCl_2 , 0.5% (wt/vol) glucose, or 0.5% (wt/vol) MAP when appropriate.

Alizarin red S staining. Plates with *Pseudomonas* strains were stained with 1% (wt/vol) alizarin red S (Sigma-Aldrich), pH 4.1, for 5 min and then rinsed with water to assay for areas of high calcium concentration after 1 day of growth on NB agar or NB swarming agar supplemented with 5 mM CaCl_2 . This experiment was repeated three times.

X-ray diffraction. *P. syringae* pv. tomato strains were grown overnight in KB to stationary phase. Two hundred microliters of each culture was plated on NB agar plates supplemented with 5 mM CaCl_2 . The strains were grown for 1 day and then desiccated with a vacuum concentrator. The desiccated cultures were ground into a powder and analyzed on a Scintag Theta-Theta X-ray diffractometer (ThermoFisher Scientific, Waltham, MA) at the Cornell Center for Materials Research, Cornell University.

Raman spectroscopy. *P. syringae* pv. tomato strains were grown as described above, and 5 μl of each strain was spotted on NB agar plates supplemented with CaCl_2 . After 1 day of growth, *in vivo* Raman spectroscopy was performed on cells using a 735-nm laser on an InVia confocal Raman microscope (Renishaw, Inc., Hoffman Estates, IL).

pH measurements on agar plates. *P. syringae* pv. tomato strains were grown as described above. Five microliters of cultures was spotted on NB agar plates with 0.01% (wt/vol) bromothymol blue (BB) that were supplemented with 5 mM CaCl₂, 0.5% (wt/vol) glucose, or 0.5% (wt/vol) MAP when appropriate. Pictures of plates were taken directly after spotting and after 1 day of growth.

Gluconic acid concentration assays. *P. syringae* pv. tomato strains were grown overnight in KB medium and then washed two times in NB medium before being resuspended at an OD₆₀₀ of 0.1 in 3 ml of NB medium, NB medium supplemented with 5 mM CaCl₂, NB medium supplemented with 0.5% (wt/vol) glucose, NB medium supplemented with 5 mM CaCl₂ and 0.5% (wt/vol) glucose, or NB medium supplemented with 5 mM CaCl₂ and 0.5% (wt/vol) MAP. Cultures were allowed to grow for 24 h before 1 ml of each culture was collected and centrifuged. The amount of gluconate/gluconic acid in each culture supernatant was determined using a D-gluconate (D-gluconic acid) assay kit (colorimetric) (Sigma-Aldrich) according to the manufacturer's directions. This assay was repeated using three biological replicates that were collected during three independent experiments.

RNA extraction. RNA was extracted from *P. syringae* pv. tomato in the same manner and under the same conditions as described previously (3).

Reverse transcription-PCR. RNA extracted from *P. syringae* pv. tomato was reverse transcribed into cDNA using a Bio-Rad iScript kit (Bio-Rad, Hercules, CA). Additionally, a reaction without reverse transcriptase (RT) was performed for each sample. *P. syringae* pv. tomato genomic DNA (gDNA), cDNA, and the no-RT controls were amplified using NEB OneTaq (New England Biolabs, Ipswich, MA) with the primers oMRF0106 and oMRF0065. PCR products were run on a 2% (wt/vol) agarose gel and visualized using a ChemiDoc XRS system (Bio-Rad).

Creation of PSPTO_5255, PSPTO_5256, and PSPTO_5255/5256 mutants. *P. syringae* pv. tomato strains with a clean, in-frame deletion of PSPTO_5255, PSPTO_5256, or PSPTO_5255/5256 were generated using pK18mobsacB (48). DNA fragments approximately 0.9 kb and 1.0 kb upstream and downstream of PSPTO_5255 or PSPTO_5256 that included the beginning and end of the gene were PCR amplified. The PCR products were gel purified using a Zymoclean gel extraction kit (Zymo, Irvine, CA). These PCR products were then inserted into pK18mobsacB as described previously, and the generated plasmids were used to create the clean, in-frame deletions in *P. syringae* pv. tomato as described previously (49).

Construction of strains constitutively expressing PSPTO_5255, PSPTO_5256, and PSPTO_5255/PSPTO_5256. PSPTO_5255, PSPTO_5256, or PSPTO_5255 and PSPTO_5256 were PCR amplified using the Q5 DNA polymerase (NEB), and the PCR products were purified using a Zymo DNA Clean and Concentrator-5 (Zymo) kit. The PCR products were inserted into a pENTR/SD/D/TOPO vector (ThermoFisher Scientific) and transformed into TOP10 *E. coli* cells. Plasmids containing the correct insert were determined, and the insert was shuttled to the destination vector pBS46 and then into ΔcvsS, ΔcvsR, Δ5255, or Δ5256 cells using a previously described protocol (50).

Construction of PSPTO_5255 luciferase reporter gene construct. The area 250 bp upstream of the annotated translational start site of 5255 was amplified by PCR using Phusion DNA polymerase (ThermoFisher Scientific). The PCR products were inserted into a pENTR/D/TOPO vector and transformed into TOP10 *E. coli* cells. Plasmids containing the correct insert were determined, and the insert was shuttled to the destination vector pBS58 using a previously described protocol (50). pBS58:P₅₂₅₅ was transformed into *P. syringae* pv. tomato through electroporation. Transformants were selected using KB agar plates with 10 μg/ml tetracycline and 50 μg/ml kanamycin and verified through PCR.

Luciferase assays. Luciferase assays for *P. syringae* pv. tomato pBS59::P_{cvsSR} and *P. syringae* pv. tomato pBS58::P₅₂₅₅ were used as a proxy for transcription of cvsS and cvsR or 5255 as previously described (3). These assays were independently repeated three times and statistically analyzed using least squares mean regression.

Statistical analysis. Jmp Pro, version 12, was used to perform statistical analyses.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00584-18>.

SUPPLEMENTAL FILE 1, PDF file, 3 MB.

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We have no conflicts of interest to declare.

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