CytR Homolog of *Pectobacterium carotovorum* subsp. *carotovorum* Controls Air-Liquid Biofilm Formation by Regulating Multiple Genes Involved in Cellulose Production, c-di-GMP Signaling, Motility, and Type III Secretion System in Response to Nutritional and Environmental Signals

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*Pectobacterium carotovorum* subsp. *carotovorum* [Pcc (formerly *Erwinia carotovora* subsp. *carotovora*)] PC1 causes soft-rot disease in a wide variety of plant species by secreting multiple pathogenicity-related traits. In this study, regulatory mechanism of air-liquid (AL) biofilm formation was studied using a cytR homolog gene deletion mutant (ΔcytR) of Pcc PC1. Compared to the wild type (Pcc PC1), the ΔcytR mutant produced fragile and significantly (*P* < 0.001) lower amounts of AL biofilm on salt-optimized broth plus 2% glycerol (SOBG), yeast peptone dextrose adenine, and also on King’s B at 27°C after 72 h incubation in static condition. The wild type also produced significantly higher quantities of AL biofilm on SOBG-Mg⁻ (magnesium deprived) containing Copper (Cu²⁺), Zinc (Zn²⁺), Manganese (Mn²⁺), Magnesium (Mg²⁺), and Calcium (Ca²⁺) compared to the ΔcytR mutant. Moreover, the wild type was produced higher amounts of biofilms compared to the mutant while responding to pH and osmotic stresses. The ΔfliC (encoding flagellin), ΔflhD::Tn5 (encoding a master regulator) and ΔmotA (a membrane protein essential for flagellar rotation) mutants produced a lighter and more fragile AL biofilm on SOBG compared to their wild counterpart. All these mutants resulted in having weak bonds with the cellulose specific dye (Calcofluor) producing lower quantities of cellulose compared to the wild type. Gene expression analysis using mRNA collected from the AL biofilms showed that ΔcytR mutant significantly (*P* < 0.001) reduced the expressions of multiple genes responsible for cellulose production (*bcsA*, *bcsE*, and *adrA*), motility (*flhD*, *fliA*, *fliC*, and *motA*) and type III secretion system (*hrpX*, *hrpL*, *hrpA*, and *hrpN*) compared to the wild type. The CytR homolog was therefore, argued to be...
INTRODUCTION

Pectobacterium carotovorum subsp. carotovorum [Pcc (formerly Erwinia carotovora subsp. carotovora)] is a Gram-negative, necrotrophic and opportunistic phytopathogenic enterobacterium. It is responsible for causing soft-rot on a variety of plant species during cultivation and, in many cases, during post-harvest processing and storage of crops, resulting in significant economic loss (Davidsson et al., 2013). Production of plant cell-wall-degrading enzymes, including pectate lyases (Pel), cellulases (Cel), and proteases (Prt) is the most destructive feature of this pathogen (Toth et al., 2003). Its pathogenicity is also known to be influenced by quorum sensing (Charkowski et al., 2012), motility (Matsumoto et al., 2003; Hossain et al., 2005), type III secretion system (T3SS) encoded by the hypersensitive response and pathogenicity (hrp) gene cluster (Rantakari et al., 2007), gluconate metabolism (Mole et al., 2010), the magnesium/nickel/cobalt transport system (Kersey et al., 2000), the biosynthesis of pyrimidine, purine, leucine, or serine (Lee et al., 2013) and biofilm formation (Lee et al., 2013). These pathogenicity factors in Pcc have been shown to be controlled by different regulatory proteins, such as KdgR (Liu et al., 1999), PehR-PehS (Flego et al., 2000), Expl-ExpR (Lee et al., 2013), GacA-GacS (Cui et al., 2001), RsmA-RsmB-RsmC (Chatterjee et al., 2002a), PmrA-PmrB (Hyytiäinen et al., 2003), and CytR (Matsumoto et al., 2003) in a complex manner.

Biofilms are surface-associated microbial communities, and are able to grow in different abiotic and biotic surfaces (Dolan and Costerton, 2002; McDougal et al., 2012). Biofilm development in bacteria can be divided into three distinct stages (Kaplan, 2010) viz., adhesion, multiplication, and dispersion. In the first stage, planktonic bacteria usually adhere to a biotic or abiotic surface either by physical process including van der Waals forces and electrostatic interactions, or by bacterial surface appendages such as flagella and pili (Rosen and Lamont, 2000). In the second stage, bacteria multiply and communicate with each other through cell-cell communication (auto-inducer) signals (McDougal et al., 2012). At this stage, they also secrete a slimy gelatinous material to form a sizable biomass (Kaplan, 2010). This bacterial biomass comprises of exopolysaccharides (EPSs [mainly cellulose]), proteins, and extracellular DNA (Sutherland, 2001; White et al., 2003; Yap et al., 2005; Liang et al., 2010). The contents ultimately determine the architecture and the stability of a biofilm biomass (Koechler et al., 2015) although their quantities vary depending on the bacterial strains and environmental conditions (Zogaj et al., 2001; Solano et al., 2002; Haque et al., 2012). The sessile bacterial biomass are known to have a hetero-dimensional structure (Oliver et al., 2014) with micro-channels and extended limbs to trap nutrients. The whole assembly acts as a protective barrier against toxins, antimicrobial agents, and predators. In the final stage, a mature biofilm often experiences detachment of some of its own biomass. It is signaled either by the bacteria themselves or caused by external forces (Kaplan, 2010). Such dispersal are considered to be a survival mechanism (O’Toole et al., 2000; Solano et al., 2002; Teitzel and Parsek, 2003; Scher et al., 2005; Haque et al., 2012) as the detached biofilms travel to colonize other sites leading to virulence (Elkins et al., 1999; Yildiz and Schoolnik, 1999; van Houdt and Michiels, 2005; Jahn et al., 2008; Yang et al., 2008; Haque et al., 2012).

Cultured bacteria generally form three types of biofilms in the laboratory: solid-air-liquid (SAL) interface biofilm, solid-air (SA) interface biofilm, and air-liquid (AL) interface biofilm. The AL biofilm type is known as pellicle (Zogaj et al., 2001; Solano et al., 2002; Yap et al., 2005; Haque et al., 2009). They have separate genetic, chemical, and cultural distinctions (Friedman and Kolter, 2004; Yap et al., 2005; Yang et al., 2008; Haque et al., 2012). Formation of AL biofilm is prevalent in numerous Gram-negative aerobic or facultative aerobic bacteria (Armitano et al., 2014). Flagella, different types of pili, curli fimbriae, type I secretion system, T3SS, cellulose, lipopolysaccharide, and quorum sensing are important for AL biofilm formation (Davey and O’Toole, 2000; Yap et al., 2005; Anriany et al., 2006; Jahn et al., 2008; Liang et al., 2010; Haque et al., 2012; Yamamoto et al., 2012). Nutritional (such as media composition, carbon sources and divalent cations particularly, magnesium, calcium, and iron) and environmental conditions (such as temperature, oxygen tension, chemotaxis, pH, and osmolarity) are also important determinants of the development of AL biofilm in bacteria (Yap et al., 2005; Liang et al., 2010; Yamamoto et al., 2011; Haque et al., 2012; Armitano et al., 2013). Furthermore, numerous transcriptional factors such as AgfD in Salmonella typhimurium (Römling et al., 2000), SpoOA in Bacillus subtilis (Branda et al., 2001); SlyA, PhoP-PhoQ two component regulatory system (TCS) in Dickeyadadantii (formerly Erwinia chrysanthemi) 3937 (Haque et al., 2009, 2012), Bcam1359 in Burkholderia cenocepacia H111 (Fazli et al., 2011), QseC in Escherichia coli (Hadjifrangiskou et al., 2012) and RscS in Vibrio fisheiri (Yip et al., 2006) were also found to have regulated the AL biofilm formation in response to different environmental and nutritional cues.

CytR (Cytidine Repressor) is known as a transcriptional repressor of nucleoside uptake and catabolism genes in some bacterial species such as E. coli (Valentin-Hansen et al., 1996),
Salmonella enterica serovar Typhimurium (Thomsen et al., 1999) and Vibrio cholerae (Haung and Watnick, 2002). Biofilm formation in V. cholerae is controlled by the CytR through the repression of vps genes, which encode enzymes essential for EPS production (Haung and Watnick, 2002). Watve et al. (2015), on the other hand, reported that CytR is a global positive regulator of competence, type VI secretion and chitinases in V. cholerae. In case of Pcc PC1 (formerly EC1) however, the CytR homolog has only been partially characterized by Matsumoto et al. (2003). They showed that the ΔcytR mutant is able to reduce the polygalacturonase (Peh) production and increase the production of Pel, Cell, and Ptl with respect to its wild counterpart. Swimming motility and the expression of fliA (encoding ßβß) and fliC (encoding flagellin) were found to have been dramatically reduced unlike flhD (encoding a master regulator) in the ΔcytR mutant. Consequently, the virulence was radically reduced in the ΔcytR mutant compared to that of the parental strain (Matsumoto et al., 2003). Such discovery was supplemented by Hossain and Tsuyumu (2006) who reported an instance of Pcc PC1 forming SAL biofilm in microtiter plates [made of polyvinyl chloride (PVC)] containing yeast extract peptone broth plus salts of M63 minimal medium at 27°C in static condition. They also showed that SAL biofilm is controlled by motility itself. Despite their efforts, the role of CytR homolog in the formation of AL biofilm in glass test tubes is yet to be quantified under different environmental (i.e., temperature, pH, osmolarity, oxygen tension) and nutritional (i.e., media composition, carbon sources, divalent cations) conditions for Pcc PC1. In addition, the expression of certain genes in this mutant has not been explored with respect to cellulose production. 

Cellulose constitutes a gulf of the exopolymeric matrix of AL biofilm in bacteria (Yap et al., 2005; Yang et al., 2008; Haque et al., 2012). It is synthesized by bacterial cellulose synthesis proteins encoded by the bcs operons, such as bcsABCD and bcsEFG (Römling and Galperin, 2015). BcsA is an integral inner membrane protein attached to BcsB, a periplasmic protein. The BcsA contains, among others, a C-terminal fragment that consists of a cyclic-dimeric (3′→5′)-guanosine monophosphate (c-di-GMP) binding PilZ domain (Amikam and Galperin, 2006). The c-di-GMP is known to control numerous cellular functions (c-di-GMP) binding PilZ domain (Amikam and Galperin, 2006). The c-di-GMP is known to control numerous cellular functions.

The T3SS is encoded by hrp (hypersensitive response and pathogenicity) and hrc (hypersensitive response conserved) genes. Yap et al. (2005) showed that T3SS regulatory (HrpX, HrpY, HrpS, and HrpL) and effector (HrpA and HrpN) proteins are required for AL biofilm formation in D. dadantii 3937. A more comprehensive study by Yi et al. (2010) showed that T3SS and biofilm formation on plastic are mediated by phosphodiesterases (PDEs) containing GGDEF and EAL-domain proteins that affect c-di-GMP turnover in D. dadantii 3937. The Pcc PC1 genome is known to be containing several GGDEF and EAL-domain proteins. Therefore, the assumption is that such proteins might regulate the biofilm formation in Pcc PC1. Previous studies in this regard, have shed some light on the regulatory role of these genes in case of SAL biofilm only (Yi et al., 2010). Nonetheless, the scientific communities are yet to find out if these genes are able to regulate the AL biofilm formation, or if the CytR homolog of Pcc PC1 can also be affected. This study will contribute toward understanding the role of CytR homolog in cellulose production, while quantifying the expression of bcs and T3SS genes in Pcc PC1.

### MATERIALS AND METHODS

#### Bacterial Strains and Growth Media

Pectobacterium carotovorum subsp. carotovorum [formerly E. carotovora subsp. carotovora (Pcc)] PC1 [formerly EC1 (wild type)], its derivative strains, ΔcytR (aflagellated and non-motile mutant), ΔfliC (non-motile and aflagellated mutant), flhD::Tn5 (aflagellated and non-motile mutant) and ΔmotA (flagellated and non-motile mutant) and D. dadantii (formerly Erwinia chrysanthemi) 3937 (wild type) used in this study has been described earlier by Matsumoto et al. (2003), Haque and Tsuyumu (2005), and Hossain et al. (2005). The strains were freshly grown in yeast extract peptone (YP) medium (1% peptone, 0.5% yeast extract, pH 6.8) at 27°C. Nalidixic acid (30 µg/mL) and kanamycin (50 µg/mL) were added to the media when required, and the optical density (OD) of the culture was measured by a spectrophotometer (Intertech, Inc. Tokyo, Japan) at 660 nm.

#### Media, Carbon Source, and Temperature on AL Biofilm Formation

Yeast extract peptone, Luria-Bertani (LB) medium (1% of tryptone, 0.5% of yeast extract, 0.5% of NaCl, pH 7.0), Salt-optimized broth (SOB) plus 2% of glycerol (SOBG) medium (per liter: 20 g of tryptone, 5 g of yeast extract, 0.5 g of NaCl, 0.186 g of KCl, 2.4 g of MgSO4·7H2O and 2% of glycerol), King’s B (KB) medium (per liter: 10 g of peptone, 1.5 g of K2HPO4, 15 g of MgSO4·7H2O and 15 mL of glycerol), yeast peptone dextrose adenine (YPDA) medium (per liter: 20 g of yeast extract, 40 g of peptone, 40 g of glucose monohydrate, and 80 mg of adenine hemisulfate) and M63 glycerol minimal medium (per liter, 2.5 g of NaCl, 3 g of KH2PO4, 7 g of K2HPO4, 2 g of (NH4)2SO4, 0.5 mg of FeSO4, 2 g of thiamine hydrochloride, and 2 g of

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glycerol] were used. In order to quantify the impacts of media and temperature on AL biofilm formation, a single colony of the Pcc PC1 and the ΔcytR mutant was grown in shake (180 rpm) culture in YP broth at 27°C until early stationary phase (OD₆₀₀ at 1.0). Afterward, 50 µL of each culture [ca. 10⁷ colony forming unit (CFU)/mL] were suspended in glass test tubes containing 5 mL of the broth. Two separate suspensions were made and incubated at two different temperatures (27 and 37°C, respectively) for 72 h in static condition. In order to study the role of carbon source on AL biofilm formation, 2% glycerol in SOBG was replaced by 2% glucose, sucrose, or mannitol. Among the media, the Pcc PC1 produced a thick and robust AL biofilm on SOBG broth at 27°C. The SOBG media and 27°C temperature was therefore taken to study the AL biofilm unless otherwise noted in this manuscript.

Quantification of the Biomass of the AL- and SAL Biofilm
Bacterial strains formed fragile to rigid AL biofilm on SOBG broth. In order to quantify the rigid biomass, samples were prepared as follows: 72 h-old AL biofilms were gently transferred to the fresh test tubes containing 2 mL of distilled water and were vortexed with sterile glass beads. The amount of the biomass in the rigid AL biofilms (OD₆₀₀) were quantified using an UV spectrophotometer (Ultrspec 3000, Pharmacia Biotech, Cambridge, England). In case of fragile AL biofilm, 1 mL of planktonic culture was carefully collected by the pipette and OD₆₀₀ was measured. Afterward, each fragile AL biofilm was mixed with 2 mL of planktonic culture and was vortexed. The OD₆₀₀ of planktonic culture was then subtracted from the OD₆₀₀ of the planktonic culture plus biomass of the fragile AL biofilm. This would provide the amount of fragile biomass present in the AL biofilm.

The AL biofilms formed at 37°C were also quantified as described in Haque et al. (2012). In brief, the suspension was carefully removed after 72 h and washed the glass test tubes with distilled water. Afterward, 0.05% (w/v) crystal violet was added and incubated for 30 min followed by rinsing with distilled water. Bound crystal violet was eluted using 95% ethanol and the AL biofilm was quantified (OD₅₇₀) using UV spectrophotometer (Ultrspec 3000, Pharmacia Biotech, Cambridge, England).

Congo Red and Calcofluor Binding Assays
Congo red and Calcofluor binding assays were carried out as described in Haque et al. (2009, 2012) with a few modifications. Initially, each bacterial strain (Pcc PC1, ΔcytR, ΔfliC, flhD::Tn5, ΔmotA, and D. dadalti 3937) [used as sensitive control for the expression of red, dry and rough (rdar) phenotype and expression of cellulose] was grown in YP broth overnight at 27°C under shaking condition (180 rpm). Each bacterial culture was serial diluted 1:100 [ca. 10⁵ CFU/mL] and 2 µL of culture were spotted (four spot in each plate) onto SOBG agar plates containing 40 µg/mL of Congo red (Sigma-Aldrich, St. Louis, MO, United States) or 200 µg/mL of Calcofluor white (Sigma-Aldrich, St. Louis, MO, United States). The plates were then incubated at 27°C in static condition, and photographs were taken after 48 h (for Congo red binding). On the other hand, after 48 h incubation, the plates were placed under UV light (366 nm) and photographs were taken for Calcofluor binding.

Quantification of Cellulose
Amount of cellulose produced in the matrix of the AL biofilms of the bacterial strains (Pcc PC1 ΔcytR, ΔfliC, flhD::Tn5, and ΔmotA) were quantified as described in Anriany et al. (2006) with a few modifications. In brief, strains were grown at 27°C in SOBG broth for 3 days in static condition. Then 3 g (wt weight) of AL biofilm masses were gently collected with sterile spatula and were dried by freeze dryer (Eyela, Freeze dryer, FDU-830, Japan). The dry masses were mixed with 4.5 mL of acetic-nitric reagent (8:2:1 acetic acid: nitric acid: distilled water) and boiled for 20 min. The boiled mixture was then centrifuged and the supernatant was discarded. The pellet was transferred to a Corex centrifuged bottles, washed twice with sterile distilled water and dried in a clean bench. The dried pellet was mixed with 150 µL of concentrated H₂SO₄ and gently shaking for 1 h at 27°C. The amount of cellulose was determined by adding 750 µL anthrone (Sigma-Aldrich, St. Louis, MO, United States) reagent (0.2 g in 100 mL H₂SO₄). The Avicel cellulose (Sigma-Aldrich, St. Louis, MO, United States) was used as standard this case, and the cellulose were quantified at 620 nm.

Isolation of RNA
Wild type (Pcc PC1) and ΔcytR mutant were grown in SOBG broth until AL biofilm was formed. Total RNA was extracted thereafter from the associated bacteria using an RNA isolation kit (Qiagen, Hilden, Germany) and was subjected to DNase I treatment with the TURBO DNase kit (Ambion, Inc., United States) as instructed by the manufacturer. The purity and concentration of RNA was estimated using a Nanodrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, United States).

Quantitative Reverse Transcription-PCR
Primers (Table 1) were designed based on Pcc PC1 DNA sequences that can be retrieved from the following address¹. cDNA synthesis, verification of the efficiencies of the primers and PCR were carried out as described in Haque et al. (2015). The relative values of transcriptional level were calculated using the ΔΔCₜ method. The abundance of specific gene was initially normalized using 16S rRNA which was shown to be invariant using best keeper (Matsumoto et al., 2003). The relative expression ratio was calculated as the differences between the cycle threshold (Cₜ) values and was determined using the following equation:

\[
\text{Fold change} = 2^{-\Delta \Delta C_T}, \text{ where } \Delta \Delta C_T = (C_T, 16S rRNA)_{\text{mutant}} - (C_T, 16S rRNA)_{\text{wildtype}}.
\]

Radish Sprouts Attachment Assays
Attachment assays were performed as described in Jahn et al. (2008) with a few modifications. In brief, radish (Raphanus
sativus) seed (10 g) was collected from Horticulture Research Centre of Bangladesh Agricultural Research Institute, Gazipur, Bangladesh. Seeds were surface sterilized with 75 mL of 1.5% sodium hypochlorite for 30 min on a rotary shaker at 200 rpm and was followed by several washes using sterile distilled water. Single colonies of bacterial strain were inoculated into SOBG broth and were grown overnight at 27°C with agitation. It was then centrifuged before each strain was resuspended in sterile distilled water. Approximately 10⁴ CFU/mL bacteria were used. For attachment assays, seeds were germinated in sterile water for 4 days with regular water changes. The sprouts were transferred in sterile 50 mL conical tubes (10 sprouts/tube) and were incubated in bacterial suspension for 4 h at 27°C with gentle shaking (50 rpm). Sprouts were then rinsed three times with sterile distilled water and homogenized. After serial dilution, the homogenate was plated and incubated at 27°C for 24 h for colonies to be enumerated. The experiment was repeated at least three times with at least seven sprouts analyzed per strain each time.

### Stress Tolerance Tests

Stationary (OD₆₆₀ at 1.5) phase cells (grown in shaking condition) and cells-associated with AL biofilm of the wild type and the ΔcytR mutant were prepared as described in Haque et al. (2012). In order to prepare the planktonic cells (i.e., cells under the AL biofilm), 1 mL of planktonic culture was carefully collected after 72 h incubation followed by centrifugation. The pellet was then re-suspended in sterile distilled water. In order to test the sensitivities, the stationary-phase, the planktonic cells and the AL biofilm cells (ca. 10⁸ CFU/mL) of the wild type and the ΔcytR mutant were separately exposed to acidic pH 4.0 (40 mM citric acid and 20 mM dibasic sodium phosphate, pH 4.0) and oxidative stress (H₂O₂ at 10 mM) which corresponds to a lethal concentration (Haque et al., 2009). The suspensions were incubated for a further 2 h at 27°C. The number of CFU was determined by serial dilution and plating onto SOBG agar just prior to inoculation and during every 30 min over a 2-h incubation. The survival of treated cells was normalized to the number of CFU at the beginning of the test.

### Statistical Analysis

All the experiments were conducted in complete randomized design with three replications and repeated at least three times unless otherwise stated. Data were analyzed using Student’s t-test of the SAS software system 8.02 (SAS Institute, Cary, NC, United States).

## RESULTS

### Reduced AL Biofilm Formation in the ΔcytR Mutant in Different Growth Media

In order to quantify the impacts of media composition and temperature on AL biofilm formation, the Pcc PC1 and the ΔcytR mutant cells (ca. 10⁷ CFU/mL) were suspended in five different media (SOBG or KB or LB or YM or M63 glycerol minimal media) and was incubated at two different temperatures (27 or 37°C) in static condition. Selection of five different media and two different temperatures was important to generate information that previous researches did not shed light on. A study by Hossain and Tsuyumu (2006) showed that Pcc PC1 produces SAL biofilm on YM broth plus salts of M63 minimal medium in the wells of microtiter plates made of PVC at 27°C in static condition. They also examined two other media such as YM and LB broth but neither of them produced any SAL or AL biofilms in Pcc PC1. The present study particularly deals with AL biofilm formation in glass test tubes under different environmental conditions which were not examined in any other contemporary researchers dealing with Pcc PC1 and its ΔcytR mutant. This study aimed to contribute toward this area of concern, as it was carried out under different media and temperature for a comprehensive result. The results of this study showed that Pcc PC1 formed AL biofilm

### Table 1

| Primer pairs    | Sequence (5’ → 3’) | Product length (bp) |
|----------------|-------------------|---------------------|
| bcsA_forward   | TGTAGGCGTGCAAACGGAAGAA | —                   |
| bcsA_reverse   | TCACCGGTGCAAACGGAAGAA | 315                 |
| bcsE_forward   | ACAGAAGAACGCGAGGCTATCCCA | —                   |
| bcsE_reverse   | CGGATGGCGTAAAGGAGACCT | 443                 |
| adrA_forward   | TATACACCGCGTGAAAGCTG | —                   |
| adrA_reverse   | CGGAGGCCATACATCGGATTT | 285                 |
| PC1_RS16795_forward | TTTCGCGCGCGAGCTATC  | 371                 |
| PC1_RS16795_reverse | AGTGCTGCTGAAATGATCTG | —                   |
| flic_forward   | AGTGTTCTGGCGACGATATG | —                   |
| flic_reverse   | GGACAGATGATCTGTCGTC   | 295                 |
| fla_forward    | CGAATCGCGGTGAGTCTG    | —                   |
| fla_reverse    | TCACTGCTGCGAGGAGTTC   | 359                 |
| thD_forward    | TCAATGGCAGACCTAAAGCA  | —                   |
| thD_reverse    | ATGTGTCTTGGCGACGATT  | 361                 |
| motA_forward   | GATCAAAATACGCGGAAGACC | —                   |
| motA_reverse   | CTCGCGCCAGCTACGCTGAA | 295                 |
| hpxX_forward   | GGCGGCGAAAGGTGAAGTGT | —                   |
| hpxX_reverse   | ATCTCATGACCCCCCTTTGCT | —                   |
| hplL_forward   | TACCGGGAACACTGATGAC   | 294                 |
| hplL_reverse   | TACCCGTTGAAACACTGATG  | —                   |
| hps_S_forward  | CCTCGGATGAAAGGCTGTTA | —                   |
| hps_reverse    | GATGAAATGCAAGATATGCGC | 271                 |
| hprA_forward   | AGAACCTGATACGTTTGGCC  | —                   |
| hprA_reverse   | CGACGTGTCTCAGGATCTG   | 196                 |
| hprN_forward   | GGATTTCCGGCTTGCGCGCAA | —                   |
| hprN_reverse   | CTGTGTGTGTTAGCGACTGGAA | 418                |
| rpsO_forward   | CTTCTGCTGACAGTTCCCAA | —                   |
| rpsO_reverse   | GGGATAGTGTGCGCTGCCTG | 291                 |
| rsmA_forward   | CGCTTGCTGACAGTTCCCAA | —                   |
| rsmA_reverse   | TGATCCTGGACGAGTATGAGG | 253                 |
| rsmB_forward   | CTTGTTGGCGTATTAGCAGGA | —                   |
| rsmB_reverse   | CGAAGTCTGACACGATCTCC | 306                 |
| 16S_rRNA_forward | AACTCTGCAAGATAGAAGTCTG | —                   |
| 16S_rRNA_reverse | GCATCGAATTAACACCATGCT | 327                 |
on SOBG, KB, and YPDA broth after 72 h incubation only at 27°C (Figure 1A). In fact, the AL biofilm in SOBG, KB, and YPDA broth did not continue to grow after 7 days (data not shown). However, the AL biofilm in SOBG broth showed signs of detachment at day 7 (data not shown). The AL biofilms formed by Pcc PC1 on the SOBG broth were quite rigid and was denser compared to those on the KB and YPDA broth. On the other hand, biofilms on YPDA and KB by Pcc PC1 were fragile and hence, were dispersed when the samples were disturbed. AL biofilms also acquired a sizable biomass when the glycerol in glycerol minimal did not produce any SAL or AL biofilm for both of these strains under any of the temperatures (27 or 37°C) (Figure 1A). The Pcc PC1 produced a prominent SAL biofilm on SOBG, YPDA, and KB broth (Figure 1A). The other three media (YP, LB, and M63 glycerol minimal) did not produce any SAL or AL biofilm for both of these strains under any of the temperatures (27 or 37°C) (data not shown). Similar to the AL biofilms, the amount of SAL biomass formed by Pcc PC1 on the SOBG broth compared to those on the YPDA and KB broth (3.10-fold) broth compared to the ΔcytR mutant at 27°C (Figure 1B). At 37°C however, the ΔcytR mutant and its wild counterpart formed only SAL biofilms on SOBG, YPDA, and KB broth (Figure 1A). The Pcc PC1 produced a prominent SAL biofilm on SOBG broth compared to those on the YPDA and KB broth (Figure 1A). The other three media (YP, LB, and M63 glycerol minimal) did not produce any SAL or AL biofilm for both of these strains under any of the temperatures (27 or 37°C) (data not shown). Similar to the AL biofilms, the amount of SAL biomass formed by Pcc PC1 on the SOBGMg− (magnesium deprived) and SOBGMg+ (magnesium replenished) at 27°C (Figure 1B). Initially however, both wild type (Pcc PC1) and ΔcytR mutant cells grew faster in all the broths except in M63 glycerol minimal medium where the growth was slightly delayed both at 27°C (Figures 1C,D) and at 37°C (Figures 1E,F). In general, the cell growth was slightly delayed in ΔcytR mutant compared to that of the wild type in YP broth at both temperatures (Figures 1C–F). These results suggested that biofilm formation controlled by the CytR homolog of Pcc PC1 is regulated by media composition and temperature. Bacterial growth might play an important role in this case, although it is very unlikely to be amongst the major determinants.

**Divalent Cations Induced AL Biofilm Formation Controlled by the CytR Homolog**

Divalent cations such as Mg2+, Ca2+, Cu2+, Mn2+, and Zn2+ have been shown to be the inducers and stabilizers of the biofilm formation processes (Song and Leff, 2006; Liang et al., 2010; Haque et al., 2012). However, information regarding the role of such cations in the formation of AL biofilms by Pcc PC1 and its ΔcytR mutant was not available in the literature. In this study, the Pcc PC1 (wild type) and ΔcytR mutant cells were inoculated in SOBGMg− (magnesium deprived) and SOBGMg+ with 0.009 M of Mg2+, Ca2+, Mn2+, Cu2+ and Zn2+, were incubated at 27°C under shaking (180 rpm) condition. The growth rate was not significantly different between the wild type (Figure 2A) and the ΔcytR mutant (data not shown) in different incubation period for any divalent cations. Both the wild type and the mutant cells grew quickly and attained its maximum at 18 h of incubation period both in SOBGMg− and SOBGMg+ containing 0.009 M Mg2+ and Ca2+ (Figure 2A). In SOBGMg− containing 0.009 M Mn2+, Cu2+, and Zn2+ however, the growth was slow and hence, took 24 h to reach the growth maximum (Figure 2A). Interestingly, it was only after 72 h incubation (in...
static condition) both the strains developed thinner to denser AL biofilm only on SOBGMg− containing the divalent cations (Figure 2B). Unlike this, the SOBGMg− broth at 27°C did not show any AL biofilms either in the wild type or in the ΔcytR mutant (Figure 2B). Compared to the ΔcytR mutant, wild type built a denser and robust AL biofilm on SOBGMg− containing Mg2+ and Ca2+ and a thinner and more fragile AL biofilm on SOBGMg− containing Cu2+, Mn2+, and Zn2+ (Figure 2B). When quantified, the production of biomass in the ΔcytR mutant was found to be significantly ($P < 0.001$) lower than that of its wild counterpart (Figure 2C). These results clearly indicate that the CytR homolog may positively regulate AL biofilm formation in Pcc PC1 responding to divalent cations.

Reduced AL Biofilm Formation in the ΔcytR Mutant in Response to pH

During early stages of plant infection, pathogenic bacteria often confronts an acidic pH that ranges from pH 4.5 to 6.5 (Grignon and Sentenac, 1991). In order to assess whether Pcc PC1 and ΔcytR mutant thrives under acidic conditions, these strains were exposed to acidic pH ranges (Figure 3A) using malic acid on SOBG broth at 27°C. The reason behind using malic acid is to stimulate the acidity in plant apoplast that contains malate (Grignon and Sentenac, 1991). The results showed that none of the strains grew at pH 4.5 while showing slower growth at pH 5.0 and pH 5.5 under shaking condition at 27°C. Bacterial cell growth did not differ significantly between the wild type (Figure 3A) and the mutant (data not shown) at different incubation period. The wild type formed a delicate AL biofilm on SOBG broth at pH 5.0 and pH 5.5 after 120 h in static condition (Figure 3B). On the other hand, ΔcytR mutant cells did not grow at the same condition (Figure 3B). After 72 h incubation at 27°C, the wild type formed a thinner AL biofilm on SOBG broth at pH 6.0 compared to pH 7.0 (Figure 3B). In case of ΔcytR mutant, only a few cells aggregated on the top and did not cover the whole surface at pH 6.0 (Figure 3B). Our results indicated that acidic pH delayed the AL biofilm formation. The compounds and/or the genes responsible for AL biofilm formation may be not produced or expressed in acidic conditions leading to AL biofilm formation of Pcc PC1.

Reduced AL Biofilm Formation in the ΔcytR Mutant in Response to Osmolarity

Osmolarity plays a great role in biofilm formation (Solomon et al., 2005; Rinaudi et al., 2006). In order to assess how osmolarity affects the formation of AL biofilm, NaCl and D-sorbitol were
used as osmotic agents in the SOBG broth that contains 0.5 g/L NaCl. We however, tested the growth of bacterial cells under different concentrations (0.05, 0.1, 0.2, 0.3 M) of NaCl in shaking condition at 27°C. The results suggest that the growth (OD$_{660}$) of both strains was unaffected due to the changes in osmolarity except for 0.3 M NaCl. Compared to the ΔcytR mutant, a firm and intense AL biofilm was developed by the wild type on the SOBG broth containing 0.05 and 0.1 M NaCl after 72 h incubation. Unlike this, 0.3 M NaCl produced small quantities of cells by the wild type and the mutant in shaking condition (Figure 4A) and consequently, a few cells were aggregated on the surface of the standing culture (Figure 4B). Similar results were also observed when wild type and the ΔcytR mutant cells (ca. 10$^7$ CFU/mL) was suspended in glass test tubes containing 5 mL SOBG broth and incubated at 27°C in anaerobic chamber (Thermo, Inc., Portsmouth, NH, United States) for 7 days (Figure 4C). This could be due to the lack of expression of compounds responsible for AL biofilm formation as stated by Gerstel and Römling (2001).

**Motility Itself, But Not Presence of Flagella, Is Required for AL Biofilm Formation**

Flagella-mediated motility was shown to play an important role in AL biofilm formation (Jahn et al., 2008; Yang et al., 2008; Haque et al., 2012). On the other hand, Hossain and Tsuyumu (2006) showed that motility, but not the presence of flagella, is required for SAL biofilm formation in microtiter plates of Pcc PC1. Because CytR homolog positively regulates flagella-mediated motility (Matsumoto et al., 2003), we examined whether CytR homolog of Pcc PC1 also regulates AL biofilm by controlling the motility itself. In order to confirm whether the motility itself or the presence of flagella is required for AL biofilm formation on SOBG broth at 27°C in stationary condition, we used three aflagellated and non-motile mutants such as ΔcytR, ΔfliC and flhD::Tn5, and one flagellated and non-motile mutant such as ΔmotA (essential for flagellar rotation but not required for flagellar assembly) of Pcc PC1. All the mutants produced a lighter and fragile AL biofilm compared to their wild counterpart after 72 h incubation at 27°C (Figure 5A). The results also showed

![FIGURE 3](image_url) 

**FIGURE 3** | Effect of pH on growth and AL biofilm formation in SOBG broth at 27°C. (A) Growth of wild type (Pcc PC1) at indicated pHs. (B) AL biofilm formation by the wild type (Pcc PC1) and the ΔcytR mutant. The values are mean and error bars indicate standard deviations (+) of three independent experiments.
that AL biofilm was not increased in the mutants even after longer incubation period (data not shown). When the biofilm biomass was quantified, wild type (Pcc PC1) was found to have produced significantly ($P < 0.001$) more (2.9-, 5.5-, 4.5-, and 9.6-fold) AL biofilms than that of cytR, ΔfliC, flhD::Tn5, and ΔmotA, respectively (Figure 5B). These data, therefore concludes that motility itself, but not the presence of flagella, is required for AL biofilm formation which the CytR homolog of Pcc PC1 is able to control.

Reduced Cellulose Production in the ΔcytR, ΔfliC, flhD::Tn5 and ΔmotA Mutant

Biofilm producing bacteria develop red, dry and rough (rdar) phenotype (also known as rugose/wrinkled phenotype) on Congo red agar plates (Römling, 2005; Haque et al., 2012; Milanov et al., 2015). We hypothesized that CytR homolog, FliC, FlhD, and MotA may affect the expression of rdar phenotype. The results of this study show that the expression of rdar phenotype on Congo red agar plates was indistinguishable between the mutants (such as ΔcytR, ΔfliC, flhD::Tn5, and ΔmotA) and the wild type (Figure 5C). Therefore, the rdar phenotype may not be controlled by the CytR homolog, FliC, FlhD, and MotA in Pcc PC1.

It is understood that rdar expressing bacteria usually binds with the cellulose specific dye Calcofluor (Zogaj et al., 2001; Solano et al., 2002; Römling, 2005; Uhlíčk et al., 2006; Steenackers et al., 2012; Milanov et al., 2015). We therefore evaluated whether wild type Pcc PC1 and the mutants (ΔcytR, ΔfliC, flhD::Tn5, and ΔmotA) also binds to Calcofluor. The results showed that the wild type (Pcc PC1) had induced bright fluorescence similar to D. dadantii 3937, while all the mutants (ΔcytR, ΔfliC, flhD::Tn5, and ΔmotA) weakly induced bright fluorescence (Figure 5D). This result indicated that wild type Pcc PC1 may produce more cellulose-rich EPS compared to the mutants.

Because cellulose production and AL biofilm formation are correlated in bacteria, we quantified cellulose production in the matrix of the biofilms. The wild type was found to have produced...
different strains. The mean values and standard deviations were calculated for each strain. The values were compared using one-way ANOVA followed by Tukey’s post-hoc test.

Figure 5: 
(A) P. carotovorum PC1 with CytR, Δflc, ΔfliC, ΔflhD::Tn5, and ΔmotA mutant strains. 
(B) Quantification of biomass biofilms in the wild type and the mutants after 48 h incubation. 
(C) Congo red binding (SOBG agar plates containing 40 µg/mL Congo red) abilities of the wild type and the mutants after 48 h incubation. 
(D) Calcofluor binding (SOBG agar plates containing 200 µg/mL Calcofluor) capacities of the wild type and the various mutants after 48 h incubation. These experiments were repeated at least three times. The values are mean and standard deviation of three independent experiments.
mutant (Figure 6) compared to its wild type. However, the expression of PC1_RS16795 (a EAL domain protein) had a dramatic increase (2.7-fold) in the ΔcyrR mutant compared to the wild type. Thus, CytR homolog may control the GGDEF (AdrA) and EAL (PC1_RS16795) domain proteins in Pcc PC1.

Reduced Expressions of Motility- and T3SS Genes in the ΔcyrR Mutant

Because ΔflhD, ΔfliC, and ΔmotA produced significantly the lower amount of cellulose than their wild counterpart, in this study, expressions of flhD, fliA, fliC, and motA were measured by quantitative reverse transcription-PCR using mRNA collected from the AL biofilms formed by the wild type and its ΔcyrR mutant. Expressions of flhD (2.3-fold), fliC (11-fold), fliA (8.3-fold), and motA (16.7-fold) were significantly (P < 0.001) decreased in the ΔcyrR mutant than the wild type (Figure 6). These results indicated that CytR homolog positively controls the expressions of flhD, fliA, fliC, and motA in Pcc PC1.

In D. dadantii 3937, T3SS regulatory (hrpX, hrpY, hrpS, and hrpL), structural (hrpA, hrcI) and effector (hrpN) genes are shown to be required for AL biofilm formation (Yap et al., 2005). GGDEF and EAL domain proteins in D. dadantii 3937 were also shown to be negatively regulated both the T3SS and biofilm formation (Yi et al., 2010). In our study, we found that GGDEF and EAL domain proteins are also controlled by the CytR homolog of Pcc PC1 (Figure 6). Thus, the expression of T3SS genes was examined by quantitative reverse transcription-PCR using mRNA collected from AL biofilms of the wild type and ΔcyrR mutant. Compared to the wild type, the expression of hrpX (2.8-fold), hrpL (4.3-fold), hrpA (4.8-fold) and hrpN (7.1-fold) was found to have significantly (P < 0.001) reduced in the ΔcyrR mutant (Figure 6). Thus, CytR homolog is positively controlled the hrp genes in Pcc PC1. These results also suggested that CytR homolog is required for hrpL expression, which in turn activates the expression of hrp genes in the HrpL regulon in Pcc PC1 (Chatterjee et al., 2002b; Yap et al., 2005; Yi et al., 2010).

In our experiment however, the expression of hrpS did not differ significantly between the wild type and its ΔcyrR mutant (Figure 6). CytR homolog may affect the expression of hrpL through the known hrpL regulators, such as RpoN (σ54), RsmA (a small RNA-binding protein) or RsmB (a regulatory RNA that binds to and sequesters the negative effect of RsmA on hrpL mRNA by forming RsmA–RsmB complex) (Chatterjee et al., 2002b; Yi et al., 2010). The amount of rsmA and rsmB transcripts in ΔcyrR mutant was similar to that in the wild type (Figure 6). Compared to the wild type, the expression of rpoN was significantly (P < 0.001) lower (3.7-fold) in the ΔcyrR mutant (Figure 6). Therefore, the CytR homolog may be termed as a regulator of hrpL expression which it achieves by altering the expression of rpoN in Pcc PC1.

Reduced Attachments to Radish Sprouts by ΔcyrR, ΔfliC, flhD::Tn5, and ΔmotA Mutants

Because CytR homolog can regulate AL biofilm formation, we hypothesized that similar genes were also required for
attachment to plant tissues as well. The ΔcytR mutant was significantly reduced ($P < 0.001$) in attachment to radish sprouts compared with the wild type (Figure 7). We also tested the ΔfliC, flhD::Tn5, and ΔmotA mutants for the attachment. Compared to the wild type, their attachment was dramatically reduced ($P < 0.001$) in these mutants (Figure 7). Thus, CytR homolog, FliC, FlhD, and MotA may regulate both AL biofilm formation in culture and also during attachment to plants in Pcc PC1.

**CytR is Required for Survival in Unfavorable Environments**

When Pcc PC1 infects a plant, it confronts an unfavorable environment such as acidity and oxidative stresses. These conditions may play a vital role in survival and expression of the virulence factors (Toth et al., 2003) of any organism. Since biofilms play an important role in the survival of bacteria, we compared the sensitivities of AL biofilms and other cells under conditions of acidity (pH 4.0) and oxidative stress generated by 10 mM H$_2$O$_2$. Results suggested that the biofilm cells were more resistant than the planktonic and stationary-phase cells (Figure 8). The wild type (Pcc PC1) was found to have shown more resistance compared to the ΔcytR mutant under acidic pH (Figure 8A). All the planktonic- and stationary-phase cells of the ΔcytR mutant were killed by 10 mM H$_2$O$_2$ within the first 60 min, and only 9% of the bacteria-associated with AL biofilm of the ΔcytR mutant survived within 60-min exposure time (Figure 8B). On the contrary, a 60-min exposure of stationary-phase, planktonic and AL biofilm cells of the wild type (Pcc PC1) against 10 mM H$_2$O$_2$ resulted in 4, 13, and 45% survival, respectively (Figure 8B). Thus, formation of AL biofilm controlled by the CytR homolog may play a great role in the survival of Pcc PC1 under unfavorable environments.
FIGURE 9 | Model of CytR homolog regulatory cascade in Pcc PC1. Environmental (temperature, pH, and osmolarity) and nutritional (growth media and divalent cations) signals modulate CytR expression. The CytR homolog directly controls the expressions of bcs operons and adrA (a GGDEF domain protein), responsible for cellulose production. The adrA may produce c-di-GMP that binds to bcsA (Amikam and Galperin, 2006) and bcsE (Fang et al., 2014) leading to increase cellulose production. The CytR homolog also regulates the T3SS, essential for AL biofilm formation. The RpoN and HrpL may play a vital role in c-di-GMP regulation of T3SS. However, molecular mechanism by c-di-GMP regulate cellulose production and T3SS yet to be examined. The CytR homolog positively controls the expressions of flagellar formation and rotation genes. These genes are required for cellulose production and motility.

DISCUSSION

In this article, we demonstrated that CytR homolog of Pcc PC1 is able to positively regulate the AL biofilm formation while responding to different environmental and nutritional signals (Figures 1–5). The results also showed that the CytR homolog is capable of controlling the expressions of multiple genes required for cellulose biosynthesis, c-di-GMP signaling, motility and T3SS in Pcc PC1 (Figure 6). All the mutants were found to diminish their capacity to attachment to radish sprouts (Figure 7). We also demonstrated that AL biofilm cells of the wild type was resistant than stationary-phase and planktonic cells to acidity and oxidative stress compared to the same cells of the ΔcytR mutant (Figure 8). Therefore it can arguably be said that CytR homolog is able to positively control numerous cellular functions in Pcc PC1.

The ability of a bacteria to form biofilms depend on the nutritional conditions (Yap et al., 2005; Hossain and Tsuyumu, 2006; Rinaudi et al., 2006). Divalent cations, particularly Cu$^{2+}$, Ca$^{2+}$, Mn$^{2+}$ and Zn$^{2+}$ and Mg$^{2+}$ play a key role in this process as have been explained for Shewanella oneidensis (Liang et al., 2010), V. cholerae (Kierek and Watnick, 2003), and Pseudomonas fluorescens (Song and Leff, 2006). We also reported that low concentration of Mg$^{2+}$ significantly increase the AL biofilm formation of D. dadantii 3937 in a PhoP-PhoQ dependent manner (Haque et al., 2012). The present study also confirms that the formation of AL biofilm controlled by the CytR homolog in Pcc PC1 is activated by the different divalent cations, e.g., Mg$^{2+}$, Ca$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, and Mn$^{2+}$ (Figure 2B). Biofilm formation also depends on the environmental conditions such as temperature (Yap et al., 2005), osmotic stress and acidity (Rinaudi et al., 2006). In this study, we observed that high temperature, acidic pH, high osmolarity and anaerobic condition negatively affect the AL biofilm formation controlled by the CytR homolog of Pcc PC1 (Figures 1, 3–5). Understandably, lower production of cellulose could lead to such scenario, or the genes required for cellulose synthesis were not expressed under those extreme conditions.
Cellulose and curli are two major components of the AL biofilm in *Enterobacteriaaceae* and their traces can be determined by Congo red binding assays (Steenackers et al., 2012; Milanov et al., 2015). For example, bacterial strains expressing both curli and cellulose leads to the rdar phenotype, only cellulose produces the *pink, dry and rough* (pdar) phenotype, and only curli triggers the *brown, dry and rough* (bdar) phenotype. A less-prominent phenotype on Congo red agar: *ras* (*red and smooth; curli only), pas (*pink and smooth, cellulose only) and bas (*brown and smooth; curli only) has also been reported in some *E. coli* strains (Bokranz et al., 2005). In our study, expression of the rdar phenotype was indistinguishable between the wild type (*Pcc* PC1) and the mutants (*ΔcytR, ΔfliC, flhD::Tn5, and ΔmotA*) (**Figure 5C**). Compared to the wild type, all the mutants (*ΔcytR, ΔfliC, flhD::Tn5, and ΔmotA*) exhibited a weak bond with cellulose specific (Calcofluor) dye (**Figure 5D**). This study also revealed that cellulose production was significantly reduced in the mutants of *ΔcytR*, *ΔfliC*, *flhD::Tn5*, and *ΔmotA* compared to their wild counterpart. The present study also showed that expressions of *flhD*, *fliA*, *fliC*, and *motA* are positively controlled by the CytR homolog of *Pcc* PC1 (**Figure 6**). Thus, fragile and lower AL biofilm formation in these mutants may not be due to only the reduction of motility but also the lower production of cellulose.

Bacterial cellulose synthesis is regulated on both transcriptional and post-transcriptional levels. Expressions of bcs genes are controlled by the different regulatory proteins in different bacteria. Transcriptional regulators MlrA and CsgD of *S. enterica* serovar Typhimurium were shown to modulate cellulose biosynthesis indirectly by regulating expression of c-di-GMP synthases DGCs and phosphodiesterases (PGEs) (Römling, 2005; Römling et al., 2013). In *E. coli*, AdrA (a GGDEF domain protein) is responsible for cellulose production (Römling et al., 2000; Zogaj et al., 2001). In our present study showed that expressions of *bcsA*, *bcsE*, and *adrA* are positively controlled by the CytR homolog of *Pcc* PC1 (**Figure 6**). Thus, CytR homolog may directly regulate cellulose biosynthesis by transcriptional control of *bcsA*, *bcsE*, and *adrA* in *Pcc* PC1.

AL biofilm formation is regulated by different transcriptional proteins in bacteria. Yang et al. (2008) reported a delay in AL biofilm (pellicle) formation in *D. daadantii* mutant of *D. daadantii* 3937 due to lower expression of *hrpL, dspE* (effector protein), *hrpA*, and *hrpN*. We, in our previous study, reported that PhoP-PhoQ two component system regulates AL biofilm (pellicle) formation by controlling *bcsABCD, adrA* and *fliC* operons but not by controlling the *hrp* genes in *D. daadantii* 3937 (Haque et al., 2012). The present study showed that expressions of *hrpx*, *hrpL*, *hrpA*, and *hrpN* are positively controlled by the CytR homolog of *Pcc* PC1 (**Figure 6**). We also observed that the expression of T3SS genes is regulated by HrpL and possibly by RpoN (**Figure 6**). Based on our results, we therefore, proposed a model in **Figure 9**. It shows that the expression of motility, T3SS and cellulose producing genes are tightly controlled by the CytR homolog of *Pcc* PC1 through a sophisticated regulatory cascade (**Figure 9**). In our study, the expression of *adrA* (a GGDEF domain protein) is positively controlled by the CytR homolog of *Pcc* PC1 (**Figure 6**). It was reported that AdrA directly binds to the cellulose synthase complex to deliver c-di-GMP straight to its BcsA (Kader et al., 2006; Morgan et al., 2014). Recently, it was shown that GIL, a new c-di-GMP binding domain protein binds to BcsE which transfers c-di-GMP to BcsA indirectly (Fang et al., 2014). In line with this idea, our results also suggest a strong possibility that c-di-GMP was synthesized by *Pcc* PC1 and was controlled by the CytR homolog which lead to the production of cellulose and expression of T3SS (**Figure 9**). Similar results have been reported regarding *D. daadantii* 3937 by Yi et al. (2010) who showed that the c-di-GMP turnover was mediated by GGDEF and EAL domain proteins in *D. daadantii* 3937. However, the exact mechanism of c-di-GMP turnover in *Pcc* PC1 has yet to be deciphered.

AL biofilm formation by plant pathogenic bacteria has been shown to be positively related with their virulence in plants (Jahn et al., 2008; Yang et al., 2008; Haque et al., 2009, 2012). Jahn et al. (2011) reported that symptom development and growth in *Nicotiana benthamiana* are not different between *D. daadantii* 3937 wild type and the bcsA (a cellulose synthase) mutant. In this experiment, the degree of maceration (in potato tuber) was significantly increased following inoculation of the wild type compared to the ΔcytR mutant (data not shown). However, in *planta* Pel, Cel, and Prt production were indistinguishable between the wild type and the ΔcytR mutant (data not shown). Thus, CytR homolog may positively regulate the virulence not by controlling the plant cell-wall-degrading enzymes in *Pcc* PC1.

Several genes have been shown to be important for both AL biofilm formation and bacterial attachment to plants (Barak et al., 2005; Jahn et al., 2008). We used radish sprouts to test bacterial adherence to plants because *Pcc* PC1 is known to cause sprout rot of radish. The study showed that the ability of mutants, i.e., ΔcytR, ΔfliC, flhD::Tn5, and ΔmotA to adhere themselves to plant tissues was significantly lower than the wild type (**Figure 7**). It has been reported that cellulose synthesized by pathogens and symbionts is essential for host colonization and survival in stress conditions (Augimeri et al., 2015). We have previously reported that bacteria-associated with pellicle/AL biofilm except for aerobically grown logarithmic- and stationary-phase cells of the *D. daadantii* 3937 are more resistant to survival in acidic pH (4.0), oxidative stress and high osmolarity (Haque et al., 2012). In this study, we observed that stationary-phase-, planktonic-, and AL biofilm cells of the wild type are more resistant compared to that in the same cells of the ΔcytR mutant under adverse conditions including acidic pH (4.0) and oxidative stress generated by 10 mM H$_2$O$_2$ (**Figure 8**). These results indicate that cellulose production is linked with the bacterial survival mechanism. Higher cellulose production in the wild type is therefore associated with its increased resistance to acidity and oxidative stress. On the contrary, lower resistance in the ΔcytR mutant is linked with lower production of cellulose. This study also reported that planktonic cells are more resistant than stationary-phase cells (**Figure 8**). Similar to Matsumoto et al. (2003), we were unable to complement the restoration of the particular phenotype when cytR homolog on pPLAFR3 (tetracycline resistant, low-copy-number plasmid) or on pML122 (gentamicin resistant, low-copy-number plasmid) transformed into ΔcytR mutant of *Pcc* PC1. Complementation of the phenotypes in this regulatory gene is yet to be confirmed.
We do not know why ΔcytR mutant failed to complement the phenotypes. We observed that the CytR homolog of Pcc PC1 regulates numerous cellular functions interacting with different regulatory networks (Figure 9). Therefore, plasmid level of expression might be different at native levels and might cause a no complemented phenomena. Yap et al. (2005) reported that the hrpS mutant of D. dadantii 3937 failed to complement due to a second, unintended spontaneous mutation. It was also reported that subunit interference occurs in homologous proteins leading to failure of complementation (Leonhartsberger et al., 2000).

The AL biofilm formation in Pcc PC1 is an emergent property. We are yet to know what role AL biofilms play in the expression of virulence of Pcc PC1. In this study, we observed that bacteria-associated with AL biofilms are more resistant to acidic pH and oxidative stress. Thus, formation of AL biofilm in Pcc PC1 may protect the cells against unfavorable environment. Moreover, the matrix of AL biofilm of Pcc PC1 is composed of cellulose which is commercially available as a wound dressing material (Abeer et al., 2014). Further research endeavors with Pcc PC1 could look into this area of possibilities.

**CONCLUSION**

The present study has demonstrated that CytR homolog of Pcc PC1 positively regulate bacterial attachment to radish sprouts as it arguably controls the expression of numerous genes involved in cellulose production, c-di-GMP signaling, motility and the T3SS. The outcomes of this study are expected to contribute toward understanding the virulence factors, attachment to plant tissues and survival of Pcc PC1 in unfavorable environments.

**AUTHOR CONTRIBUTIONS**

All authors listed, have made substantial, direct and intellectual contribution to the work. All authors read and approved the final manuscript.

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