CYCLIC DI-GMP REGULATES EPS SECRETION, BIOFILM FORMATION, AND MOTILITY IN THE PLANT PATHOGEN ERWINIA AMYLOVORA

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ABSTRACT

CYCLIC DI-GMP REGULATES EPS SECRETION, BIOFILM FORMATION, AND MOTILITY IN THE PLANT PATHOGEN ERWINIA AMYLOVORA

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The second messenger cyclic di-GMP (c-di-GMP) is a nearly ubiquitous intracellular signal molecule known to regulate various cellular processes including biofilm formation, motility, and virulence. This intracellular concentration of this molecule is inversely governed by GGDEF-containing proteins (GCPs) and EAL-containing proteins (ECPs), which synthesize and degrade c-di-GMP, respectively. The role of this regulatory molecule in the plant pathogen and causal agent of fire blight disease, *Erwinia amylovora* was explored. In this study, it is demonstrated that *E. amylovora* contains three functional diguanylate cyclases (DGCs) that synthesize c-di-GMP: *gcp1*, *gcp3*, and *gcp5*; and two functional c-di-GMP specific phosophodiesterases (PDEs) that degrade c-di-GMP: *ecp1* and *ecp3*. C-di-GMP was not detected in the wild type strain. C-di-GMP was shown to positively regulate biofilm formation and secretion of the main expolysaccharide amylovoran and to inversely regulate swimming motility and virulence. A deletion of *ecp3* resulted in a hyper-biofilm forming phenotype. Interestingly, over-expression of *dgc1*, which contains both an EAL and a GGDEF domain, resulted in no observable phenotypic differences from the wild type strain, even though over-expression of *dgc1* increased the levels of c-di-GMP.

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INTRODUCTION

Fire blight

Fire blight is a devastating disease of plant species belonging to the rose family *Rosaceae,* specifically pome-producing members of the subfamily *Maloideae* or *Pomoideae*, which includes the apple and pear [100]. In 19th century America, this disease made commercial pear cultivation nearly impossible. In the 1870s, Thomas Burrill from the University of Illinois noted the appearance of tiny moving particles during the initial infection process in the cambium of a blighted branch, and went on to describe a sticky, brownish, half-fluid exudate from these branches that was filled with these oscillating particles [16]. Though he had initially mistaken these particles as fungal spores, by 1880 he had determined that they were bacteria. He confirmed that these bacteria were the causal agent of fire blight by showing that blighted pear and apple tissues could cause fire blight in asymptomatic tissues. In 1883, Burrill conclusively identified the first plant pathogenic bacterium and named it *Micrococcus amylovora* [17], which is now known as *Erwinia amylovora* (Reviewed in [37]). This was also the first plant pathogenic bacterium found to be spread by insects [6].

Erwinia amylovora, a Gram-negative bacterium of the family *Enterobacteriaceae*, is a devastating phytopathogen that infects *Rosaceae* plant species belonging to the subfamily *Pomoideae* [29]. Fire blight susceptible rosaceous species include *Cotoneaster*, *Cratagus*, *Pyracantha* and, of greatest economic importance, apple and pear trees. The disease is believed to have originated in North America, but it has since spread world-wide [99]. It was first observed in Europe in 1957 where it has continued to cause havoc [99]. Warm temperatures and precipitation or high humidity are among environmental conditions that can

predispose blossoming plants to infection [29]. Commercial apple cultivars grown on dwarfing rootstocks are often highly susceptible to infection. These trees are planted in close proximity to each other to increase revenue per acre. However, this practice inadvertently increases the chance of a rapid spread of infection [58]. This pathogen can infect flowers, fruits, actively-growing shoots, and rootstock crowns [60]. During blossom infection, which is the primary route of infection [29], the fire blight pathogen is disseminated to the stigma by insects or rainfall. Stigma-associated bacteria can be washed into the host nectary where a rich nutrient supply facilitates multiplication and a direct route inside the host at which time infection can be established [99, 104, 105]. Stigma-associated fire blight infections are often localized to the blossoms, but bacteria can spread to mature host tissues in a highly susceptible host. *E. amylovora* can also invade the host at weather- or insect-damaged lesions [29].

The first symptom of a fire blight infection is water soaking and wilting of the host tissue. In an *E. amylovora* infection, wilting is not caused by xylem blockage and water-flow disruption, but is rather due to parenchymal cell collapse and subsequent loss of turgidity in those affected tissues. Wilted host tissues cause the infected shoot to bend, reminding an observer of a "shepherd's crook," a phenotype associated with fire blight [4, 43]. Water-soaked and wilted tissues will be necrotic in a fire blight infection and take on a charred and black appearance, which is why the disease is commonly called fire blight [29].

The ability to migrate within the host is not a common attribute of plant pathogenic bacteria. This is evidenced in *Ralstonia solanacearum*; invasion of the vascular tissue of the host disrupts the water flow in the xylem resulting in wilting. The consequence is that *R. solanacearum* cells are not often found distant from the site of trauma [40]. Alternatively, *E.*

amylovora can be found upstream or downstream of a symptomatic area of the plant [29]. Once *E. amylovora* invades the vascular tissue of a highly susceptible host, it is fully capable of migrating from the infection site to the trunk or collar of the tree [58]. In a highly susceptible apple cultivar (Empire, Golden Delicious), *E. amylovora* can be found in symptomless tissue more than 50 cm below the inoculation site in as little as 11 days, and in the two year old rootstock of one apple cultivar (Empire) in only three weeks. If conditions are favorable, this migration and eventual collar blight can result in tree mortality in one year [58]. Perhaps the differences in depth of host penetration between *E. amylovora* and other plant pathogens could be due to the fact that *E. amylovora* does not excrete or produce cell wall-degrading enzymes as pectolytic or cellulolytic enzyme activity was not detected under any conditions studied [86]. By keeping to the *beaten path*, by not degrading the host xylem tissue before it is well past the traumatized site, the cells allow themselves greater access to the host.

The Economic Impact of Fire Blight

Fire blight is a very expensive disease for apple and pear lovers and even more of an economic burden for the orchard owners. A single fire blight epidemic in southwest Michigan in 2000 resulted in the death of over 400,000 trees and the removal of more than 1000 hectares of apple orchards, with a total economic loss estimated at $42 million. A 10% incidence of rootstock blight in a four-year-old high-density planting can result in losses up to $8,400 per hectare when the costs of tree replacement, lost investment in tree establishment and maintenance, and reduced yields over several years are considered [54].

Erwinia amylovora Motility

Man's first reaction to observing bacterial motility occurred in 1676 when Antony van Leeuwenhoek looked through a single-lens microscope and said: "I must say, for my part, that no more pleasant sight has ever yet come before my eye than these many thousands of living creatures, seen all alive in a little drop of water, moving among one another, each several creature having its own proper motion" [9]. Bacterial movement across a moist surface is called twitching or swarming motility, and is facilitated by pili or flagella, respectively. Movement through a liquid is called swimming motility and is also facilitated by flagella. A flagellum is a long, thin, and usually monomeric external filament anchored to a membrane bound basal body and rotates in helical fashion to propel the cell. In a liquid environment, motile bacteria can direct themselves toward an attractant with counter-clockwise flagellar rotation and away from a repellent in with clockwise flagellar rotation (Reviewed in [55]). Like many species of plant pathogenic bacteria, *Erwinia* are motile in liquid culture by means of two to seven peitrichous flagella per cell [68]. While plant pathogenic bacteria generally do not exhibit swimming motility within the host, these bacteria uniquely chemotax for, or move towards, dicarboxylic acid, which is found in the nectar of apple flowers [67].

Hrp and DSP genes encode *E. amylovora* virulence factors

Three groups of genes were found to be critical for disease: those involved in amylovoran production (see next section), those necessary for induction of the hypersensitive response (HR) in non-host plants called the *hrp* genes, and the disease-specific genes called *dsp* genes. The *hrp* genes are required for the regulation, secretion, and production of harpin proteins such as the pilus protein HrpA. Two other *hrp* proteins interact directly with the host cell wall to cause death, HrpN and HrpW (Reviewed in [1, 100]). Another important protein is

the 198 kDa effector molecule DspE, which is homologous to *Pseudomonas syringae* pathovar tomato avirulence protein AvrE [13, 35] and is known to interact with at least four apple proteins [56]. A precise functional role for DspE has not been identified, but it can suppress saylicylic acid-mediated host defenses and cause necrosis in host and non-host plants [15, 25]. HrpN, HrpW, and DspE, among others, are trafficked through the HrpA pilus of the type three secretion system (T3SS), which is conserved among many plant and animal pathogens including *Yersinia pestis*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Xanthomonas campestris* (Reviewed in [1, 33]).

Amylovoran production

Another hallmark of fire blight is the presence of viscous brown ooze on plant surfaces which is composed of extracellular polysaccharide (EPS) and cells. The main EPS in *E. amylovora* is amylovoran, a 1 MDa acidic polysaccharide which is composed of about 1000 repeats of galactose and glucuronic acid [46, 59, 64]. Amylovoran is also a virulence factor that allows *E. amylovora* to colonize the host and facilitate its transmission to new host tissues through birds and insects [3, 8]. It has been shown that among *E. amylovora* isolates, the amount of amylovoran produced is directly related to the virulence of that strain [3]. Strains that cannot produce amylovoran are not pathogenic [10, 36, 91, 110]. Amylovoran facilitates the formation of bacterial aggregates and may disrupt water flow through the xylem [89]. Vanneste adds that EPS may mask the pathogen-associated molecular pattern (PAMP) immune response, retain water and nutrients, promote tissue invasion, or cause water soaking and tissue collapse [99].

The 12 genes responsible for amylovoran synthesis and secretion are encoded within a 15.8 kb operon [36]. The *ams* operon is transcriptionally regulated by a conserved Rcs(A/V)BC phosphorelay system. In *E. coli*, the membrane-bound kinase RcsC receives an environmental signal and regulates the phosphorylation-dependent activation of RcsB. Activated RcsB can then bind RcsA/V, and that complex binds to the promoter region of the colonic acid synthesis genes, which are required for EPS production. In *E. amylovora*, RcsA/V is expressed at a very low level, and it has been shown that RcsC-activated RcsB binds the FA site at -578 to -501 of the *ams* operon [47, 103].

Biofilms

The importance of biofilms was first revealed in the 1940's, when Zobell and associates noticed that more bacteria resided on the surface of the sample containers than in the collected sample as planktonic cells [111]. Biofilms consist of a community of bacteria embedded in a matrix of EPS, DNA, and proteins, and their formation is a common survival mechanism among almost all bacteria. Organisms within a biofilm are afforded an increased resistance to antibiotics, myriad environmental stressors, and host defenses [21, 27, 30, 38, 61, 65, 92]. So advantageous are biofilms to a microbial community that their existence has been observed in fossil records dating back to between 3.2 and 3.4 billion years ago in hostile environments consisting of extreme temperature and pressure, such as hydrothermal oceanic vents, where biofilm-associated microbial communities still can be found today (Reviewed in [38]). The ability to form biofilms is essential for virulence in several plant pathogenic bacteria including *X. fastidiosa* and *E. amylovora* [19, 48], a topic discussed in more detail in the following paragraphs.

Cyclic di-GMP

In the early 1980s, work in Moshe Benziman's (Reviewed in [107] and [45]) laboratory was focused on the study of the enzymatic mechanism and regulation of cellulose biosynthesis in *Gluconacetobacter xylinus*, which was then called *Acetobacter xylinum*. In vitro enzyme assays of 1,4-β-D-Glucan 4-β-D-Glucosyltransferase (cellulose synthase) showed only 1% of previous cellulose synthase activity in cell culture. Benziman and coworkers discovered that enzyme activity was specifically activated by GTP, but only when an unknown enzyme was co-purified from membrane preparations [2]. The unknown regulatory protein was shown to bind to an agarose-hexane-GTP column, and after incubation with GTP and elution of the protein from the column, a previously unidentified guanylate derivative was found [74, 75]. The compound identified as bis(3'-5')-cyclic diguanylic acid (cyclic di-GMP or c-di-GMP) was found to be synthesized from 2 GTP molecules by an enzyme called a diguanylate cyclase (DGC) and found to be broken down by an enzyme called a phosphodiesterase (PDE) [76].

Enzymes containing a GGDEF domain are responsible for synthesis of c-di-GMP while enzymes containing either an EAL or HD-GYP domain are responsible for its degradation [62, 77, 80, 83, 94]. Proteins containing these enzymatic domains are found in many bacteria including Gram-negative alpha- and gamma-proteobacteria and Gram-positive bacteria, with some species like *Vibrio cholerae* containing as many as 60 such proteins [34]. Interestingly, it has been shown that the function of these proteins is retained among different species. The *Salmonella enterica* DGC AdrA has been shown to induce high c-di-GMP phenotypes in *Pseudomonas aeruginosa* and to complement the *Yersinia pestis* DGC HmsT [87, 88]. A number of predicted PDEs and DGCs from *Clostridium difficile* were recently tested for function in *V.*

cholerae [14]. It is now widely accepted that c-di-GMP functions as a central control module to regulate biofilm formation in the majority of bacteria [34].

Bacteria typically exist in either an individual, motile, planktonic state or in a sessile, biofilm state. Generally, a high level of intracellular c-di-GMP positively regulates aggregation, biofilm formation, and EPS production, while negatively regulating swimming motility and virulence [22, 41, 45, 72]. In *V. cholerae*, c-di-GMP induces biofilm formation and represses expression of the cholera toxin genes, which are important for causing disease in the host [96, 97]. The causal agent of the bubonic plague, *Y. pestis*, spreads from a biofilm in the flea to mammalian hosts, and biofilm formation is regulated by the DGC HmsT and the PDE HmsP [11]. The causal agent of cystic fibrosis, *Pseudomonas aeruginosa*, colonizes the lung tissue of a host during infection via c-di-GMP induced biofilms [42], and multiple GGDEF and EAL proteins are necessary for cellular toxicity in the tissue culture infection model [51].

A role for c-di-GMP in plant pathogenesis has been uncovered in *X. campestris*, the causal agent of black rot in cruciferous crops. The virulence of *X. campestris* is dependent upon secretion of extracellular proteinases and its EPS, xanthan gum. It also must be able to disperse from biofilms after host colonization. These processes are positively regulated by an HD-GYP domain containing PDE [69, 77]. Similar regulation has also been shown with other plant pathogenic bacteria such as *Pantoea stewartii* [49] and *Dickeya dadantii* [108].

In this study

The role of EPS production and the T3SS have been well characterized in *E. amylovora*. Moreover amylovoran, the major EPS of *E. amylovora,* is essential for disease. However, the role of c-di-GMP in *E. amylovora* has not been explored. Because biofilm formation and the

T3SS are critical components of plant infections, and because these processes are regulated by c-di-GMP in related species of plant pathogenic bacteria [108], we wondered if c-di-GMP signaling is also critical for *E. amylovora* virulence. My thesis probes this question. We identified 8 ORFs encoding putative c-di-GMP metabolizing enzymes *in silica*, and annotated them *ecp1-3* (EAL-containing protein) and *gcp1-5* (GGDEF-containing protein) (Fig. 1). No ORFs encoding the conserved HD-GYP domain were identified. We constructed plasmids that facilitate the IPTG-inducible expression of all 8 of these genes (Table 2). Two other plasmids encoding the IPTG-inducible over-expression of *qrgB*, a constitutively active GGDEF-containing protein from *V. harveyi* [101], and an active site mutant of this gene, *qrgB**, were used as positive and negative controls, respectively [102]. We also created in-frame deletions of each of these genes in the wild-type background (Table 2). These tools were used to elucidate the role of c-di-GMP in amylovoran production, autoaggregation, biofilm formation, flagellar motility, and virulence in *E. amylovora* strain Ea1189. In this study, we show that c-di-GMP promotes a sessile lifestyle in *E. amylovora* by positively regulating EPS production, autoaggregation, and biofilm formation and by negatively regulating flagellar motility and migration within host xylem tissues.

MATERIALS AND METHODS

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 2. Unless otherwise mentioned, *Erwinia amylovora* strain Ea1189 [18] and *E. coli* strains [26] were grown in glass tubes (18 by 150 mm) with Luria-Bertani (LB) at 28°C and 37°C, respectively, with agitation. Amylovoran secretion assays and autoaggregation assays were conducted in MBMA medium (3 g KH_2PO_4, 7 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 2 mL glycerol, 0.5 g citric acid, 0.03 g $MgSO_4$ per liter) amended with 1% sorbitol [7]. Induction of the *hrp* genes was facilitated by growing cultures in Hrp-inducing minimal medium (HrpMM) (8 mL 5x M9 salts, 40.55 μL 1M $MgSO_4$, 100 μL 0.1 g/mL niacin, 100 μL 0.1 g/mL thiamine, 34 μL 1 M $CaCl_2$, 2% galactose in 50 mL) (Modified from [44]). As required, antibiotics were added at the following concentrations: kanamycin at 100 μg/mL; ampicillin at 100 μg/mL; chloramphenicol at 10 μg/mL.

Bioinformatics

The search for ORFs in *E. amylovora* that contain GGDEF, EAL, and/or HD-GYP domains was carried out using the Motif Alignment and Search Tool (MAST) version 4.6.1 [5]. The location of predicted transmembrane domains was predicted using TMHMM version 2.0 [50] (Fig. 2B) and the presence and organization of conserved protein domains were predicted using Pfam version 25.0 [32] (Fig. 2A). Amino acid alignment was used to examine the conserved sequences of the EAL and GGDEF domains, and was accomplished using ClustalW in MEGA5.0 [95] (Fig. 2A).

DNA Manipulations

The sequence of *Erwinia amylovora* was obtained from the Sanger Institute (http://www.sanger.ac.uk/resources/downloads/bacteria/erwinia-amylovora.html). This sequence has now been published [85]. DNA manipulations were performed using standard techniques [81]. PCR was carried out using Phire Hot Start DNA polymerase (Finnizymes). FastDigest restriction enzymes (Fermentas) and T4 DNA ligase (NEB) were used in cloning. Native DGCs and PDEs were amplified from *E. amylovora* genomic DNA, digested with restriction enzymes, and cloned into pEVS143 [28] to make the over-expression plasmids. Additionally, the putative promoter region from -1 to -501 of the *ams* operon was amplified and cloned upstream of the *lux* operon in pBBR-*lux* [52]. All PCR products that were cloned into the vectors were aligned with the published genome of *E. amylovora* to confirm the sequence of the insertion.

Mutant construction

Unmarked deletion mutation of each gene predicted to be involved in synthesizing or degrading c-di-GMP in *E. amylovora* was carried out as written [24]. Briefly, the 1 kb chloramphenicol resistance (CmR) cassette with flanking FRT sites was amplified from pKD3 using 70mer primers encoding 20 bp of homology to the CmR cassette and 50 bp of homology with the regions immediately upstream and downstream of a particular target gene. Four 50 μL PCR reactions per target gene were used to amplify a 1.1 kb DNA segment and its size was confirmed by agarose-gel electrophoresis. 200 μL of the PCR product was digested with DpnI restriction enzyme (New England Biolabs) for one hour at 37°C to degrade any methylated DNA (genomic DNA and the pKD3 plasmid). I hypothesize that undigested plasmid was the cause of

the high CmR background encountered early on in this project. The DNA was then concentrated to 5 μL using Pellet Paint Co-Precipitant (EMD Chemicals Group, Merck).

E. amylovora cells containing pKD46 were grown overnight in LB with ampicillin and diluted one to 25 (usually 2 mL in 50 mL) in fresh LB supplemented with ampicillin and 1 mM L-arabinose to induce expression of the Lambda-red recombinase genes [24]. Cells were grown at 28°C with agitation in a 250 mL Erlenmeyer flask. After about four to six hours of induction, the culture density was OD_{600} 0.6-1.0, which is still mid-late exponential phase. The cells were made electrocompetent by centrifuging at 8000 g for 10 minutes in two 25 mL volumes in a large centrifuge at 4°C. The media was decanted and the cells were washed three times in 10 mL 10% glycerol at 4°C. Following the final wash step, cells were concentrated about 250-fold, yielding four to six aliquots of electrocompotent recombinase-expressing *E. amylovora*. These cells can be used immediately or stored at -80°C for one week. The now 50-fold concentrated 1.1 kb PCR product was then mixed in with the electrocompotent cells and incubated at 4°C for about 10 minutes. The mixture was then transferred to a cold 1 mm electroporation cuvette and electroporated. 400 μL SOC recovery medium (Invitrogen) was immediately added to the cells, and they were transferred to a 1.5 mL Eppendorf tube which was then capped and incubated at 28°C for one to two hours with horizontal agitation. After recovery, the cells were plated on LB agar plates with 10 μg/mL chloramphenicol and 100 μg/mL ampicillin. Cells with the mutation were identified by colony PCR with primers located 500 bp upstream and downstream of the mutation. Mutant detection by PCR was facilitated by the addition of 1.25% DMSO.

Table 1. Primers used in this study	
70-mer primers used to make 1.1 kb DNA segment for deletion by recombination	
ecp1.ko.us	5'-cgcgtaagtaatttgcacccgatacaggctaaaacaagagaaatgactctgtgtaggctggagctgcttc
ecp1.ko.ds	5'-atctgtcatataaaatgtgttgctcgggtaatgagattaaccgcaattttcatatgaatatcctcctta
ecp2.ko.us	5'-ccgccacgctgttaagccaggccgaacatatcagcgatctcgcctgggatgtgtaggctggagctgcttc
ecp2.ko.ds	5'-aatttcactaagaaacatcctatttcggcttaaataagtcgggctggcggcatatgaatatcctcctta
ecp3.ko.ds	5'-ttgtgaaactaaagaaagtcagcgcgcgcgagcagagcctggcgcggtgggtgtaggctggagctgcttc
ecp3.ko.us	5'-cgcttcggagagtttgcttgcgcgtcagccgttcattaaagattaagcaacatatgaatatcctcctta
gcp1.ko.us	5'-gtcatttttcgtattacgggaagtcactgtcaatgccaggcgaatgaatcgtgtaggctggagctgcttc
gcp1.ko.ds	5'-cgcaggctgacccgcgatatcttcaggtcagcccgccgcattgcactggacatatgaatatcctcctta
gcp2.ko.us	5'-cttaacggtgctggcacgttgatatttcagggcagtcacgagtaaatatagtgtaggctggagctgcttc
gcp2.ko.ds	5'-ataataagttaaatcaatgtggctggcccgcagagcagttcggttggctacatatgaatatcctcctta
gcp3.ko.us	5'-aacccggccggctggtgagcgattcatttttaccgtcctgagcctgaaaggtgtaggctggagctgcttc
gcp3.ko.ds	5'-ggacagttgttgctctgcaatgccaacccgcggtgagcgggttggcatcacatatgaatatcctcctta
gcp4.ko.us	5'-atggtgcaagattttaagattatgcctaaaaggcaccatgccggagagtagtgtaggctggagctgcttc
gcp3.ko.ds	5'-ccgttgcgcccggcgactgaccagccgcggccttgaggttgatggcggtacatatgaatatcctcctta
gcp5.ko.us	5'-ccagcacccgccaggaaagaaatggctaaaacatctatgctttaagccgagtgtaggctggagctgcttc
gcp5.ko.ds	5'-caacggcctgcggatcgccgtattgacctggaaaacatataaagcacagccatatgaatatcctcctta
Primers flanking target genes by 500 bp - used for confirming deletions	
ecp1 .5 us	5'-gggtagaagcattcagcgca
ecp1 .5 ds	5'-gaagaccacatcttgagcgg
ecp2 .5 us	5'-ggcacatagatgattatggcgcgtatacg
ecp2 .5 ds	5'-caggccacctaacggacgga
ecp3 .5 us	5'-gcagcgatggtctgacgcac
ecp3 .5 ds	5'-gcgccgggttgcagcacgtt
gcp1 .5 us	5'-gataaccacgctgctgaaaaac
gcp1 .5 ds	5'-ttcgtcagacgggattagccgc
gcp2 .5 us	5'-tcttcaccgcccattaaccg
gcp2 .5 ds	5'-aaacgtttatcgcagccatt
gcp3 .5 us	5'-cgcattgtttcgtcaacgaatg
gcp3 .5 ds	5'-cgccgacatccgccattacg
gcp4 .5 us	5'-cggctgagcattggctggcg
gcp4 .5 ds	5'-catgtactgtgccatcgccg
gcp5 .5 us	5'-agcgccgagtgcagaactac
gcp5 .5 ds	5'-cgacttccagggacagcgcg

The sequence of the PCR product obtained was then aligned to the wild type sequence to confirm that no other mutations inserted by PCR were present. At this point, candidate

colonies were grown overnight in LB with ampicillin and chloramphenicol and made electrocompotent using the modification of the protocol described above by harvesting only 3 mL of overnight culture. We then introduced a KmR plasmid, pTL17 [53], encoding an IPTG-inducible site-specific recombinase that causes recombination between two identical Flippase Recognition Target sequences, or FRT sites, leading to deletion of the antibiotic resistance gene. After one hour of recovery in SOC medium, the transformation efficiency was such that the recovering cell suspension could be streaked with an inoculating loop onto LB agar plates with 0.1 mM IPTG, ampicillin, and kanamycin. Isolated colonies were then replica plated onto LB with ampicillin and LB with ampicillin and chloramphenicol and incubated at 28°C for 24 hours. Chloramphenicol-sensitive clones were confirmed to have lost the CmR cassette by colony PCR using the same flanking primers originally used to confirm the insertion.

Determination of intracellular c-di-GMP levels

The procedure for the determination of intracellular concentrations of c-di-GMP levels using high performance liquid chromatography coupled with tandem mass spectrometry has been described in detail [12]. Briefly, overnight cultures were grown in LB and then used to inoculate 7 mL fresh media in a 25 mL Erlenmeyer flask with a starting with an OD_{600} of 0.05. After about 6-8 hours at the mentioned time and temperature, cells were harvested at mid-late exponential growth phase, an OD_{600} of about 0.8. The culture density in CFU/mL was calculated by serial dilution and colony counts on LB agar plates. From this culture, 5 mL of cells were centrifuged in 35 mL plastic centrifuge tubes at 4°C for 10 min at 8000 g. The supernatant was removed and the pellet was resuspended with 1 mL PBS and transferred to a

fresh 1.5 mL Eppendorf tube. The cell suspension was centrifuged at 10000 g for 1 minute and the PBS was aspirated. The cells were then lysed with 0.1 mL extraction buffer (40% acetonitrile-40% methanol in 0.1 N formic acid), left at -20°C for 20 minutes, and then centrifuged at 4°C for one minute at 15000 g. The debris-free liquid was then analyzed by HPLC-tandem MS-MS. Combined with a standard curve, this method allows for an accurate *in vivo* quantification of the concentration of c-di-GMP per extraction buffer. This value was normalized by CFU/mL of the cultures and then to the approximate volume of the cells as determined by microscopy of diluted mid-log phase cultures. Cells were immobilized on a 1% agarose pad and visualized by phase-contrast microscopy. The length and width of the cells was quantified using ImageJ and cell volume was approximated using the formula for calculating the volume of a cylinder, or in this instance, a rod-shaped bacillus (Volume = $pi*height*radius^2$). The average volume an *E. amylovora* cell in LB during exponential growth was determined to be $1.88*10^{-12}$ mL.

Motility assays

Swimming motility was examined on low-density LB plates with 0.3% agar. The plates were always made and used on the same day to avoid dehydration and therefore variability between trials. The plate was divided into quadrants and one quadrant was used for a size reference while the other three were used for triplicate measurements on each of three motility assays. The tip of a Pipetman 10 μL plastic tip fit for a P2 into overnight bacterial cultures and then plates were stab-inoculated with this cell-suspension coating the 10 μL plastic tip. The inoculated plates were incubated at 28°C for 20 hours. For the over-expression strains,

the low-density agar plates were amended with 1.0 mM IPTG and Km. Motility plates were photographed under white light from a Red Imaging System (Alpha Innotech) and the files were opened with ImageJ. From the "Threshold" window, each motility plate image was converted to dark background and the threshold was adjusted until the area of the colony was roughly translated to pixels. This same technique was done for the size reference sticker, and by normalizing the motility pixel area by the 1 cm^2 reference sticker, we determined the motility area (cm^2).

CPC binding assay for turbidometric quantification of EPS production

Overnight cultures were added to fresh MBMA medium [7] to a starting OD_{600} of 0.1 and grown for 20 hours at 28°C with agitation (3 mL media in a 18 by 150 mm glass tube). Our high c-di-GMP strains displayed retarded growth rates compared to the low c-di-GMP control strains, and in fact were found to be found in a dense, hyper-aggregative state at the bottom of their tubes. Therefore, I will now refer to growing over-expression strains in MBMA as the autoaggregation assay. We decided to use a combination of one part LB to three parts MBMA which was termed liquid gold (LG) medium. At this point, the OD_{600} of the cultures were taken and 1 ml of each culture was pelleted. 0.8 mL of the supernatant was transferred to a new 1.5 mL Eppendorf tube, mixed with 40 μL of 50 mg/mL CPC (cetylpyridinium chloride, Sigma), and incubated at room temperature for 10 minutes. The OD_{600} of the suspensions were normalized to the OD_{600} of the cultures. These numbers were again normalized to the value of

the vector control (pEVS141). The *ams* mutant contains a deletion of the entire *ams* operon and is unable to produce amylovoran [110] and was used as a negative control.

Congo Red binding assay

LB agar plates were prepared with 0.01% Congo Red (CR) [63]. A 10 μL aliquot of an overnight culture were spotted onto the surface of the agar and incubated at 28°C for 48 hours.

Biofilm formation

Biofilm formation in static conditions was quantified using crystal violet (CV) staining. The OD_{600} of an overnight culture was measured, and 1 mL LG medium was inoculated to an OD_{600} of 0.05. The DGC and PDE over-expression strains were grown with LB supplemented with kanamycin and 1.0 mM IPTG. This dilution was mixed and 0.5 mL was added to one well in a 48 well plate (Corning). The cultures were incubated at 28°C for 24 hours with no agitation. The media and planktonic cells were then very carefully aspirated with a 25 3/8 gauge needle. The remaining biofilms were resuspended in 1 mL PBS and transferred to a new 1.5 mL Eppendorf tube. These cells were pelleted by centrifugation at 10000 g for one minute and the PBS was aspirated. 50 μL 1% CV (in water) was added to and mixed with the pellet, and then incubated at room temperature for two minutes. The pellet was rinsed three times with PBS followed by CV elution from the pellet with 1 mL ethanol. The OD_{595} of the solution was recorded to estimate the concentration of CV. The wild-type and mutant cultures had no antibiotics or IPTG added to the media.

Biofilm formation was further analyzed under low-shear flow conditions using a flow-cell apparatus (Stovall Life Sciences, Greensboro NC) as previously described [48]. Briefly,

cultures were grown overnight in LB and resuspended in MBMA with kanamycin and 0.1 mM IPTG. The cultures were introduced into the flow cells and left undisturbed for one hour at room temperature to allow for initial surface attachment. Fresh media was then pumped through the flow cell chambers at 12 mL/hr for 48 hours at room temperature. The apparatus was configured as detailed in the manufacturer's instructions (Stovall Life Sciences). Chambers were examined using confocal microscopy (CLSM; Carl Zeiss Microimaging, GmbH). The images of the biofilms were captured using the laser-scanning microscope (LSM) image browsing software and represented in figure 7.

Virulence Assays

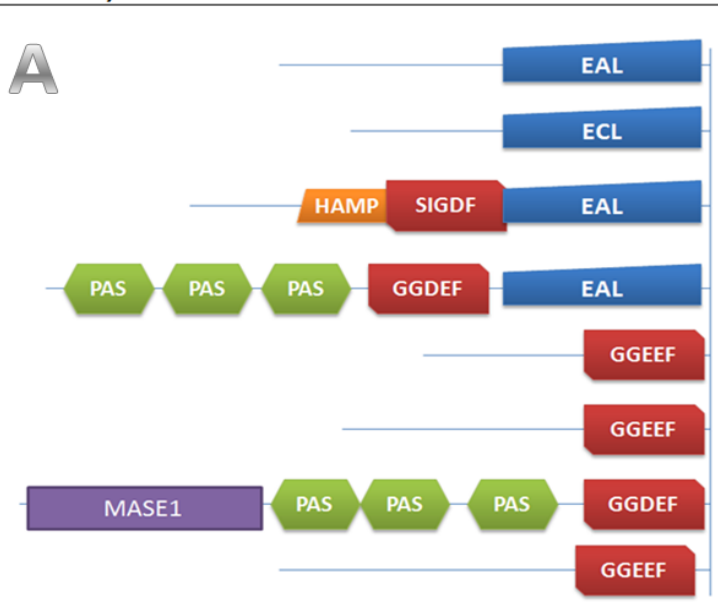
Immature pears have been used to examine the virulence phenotypes of *E. amylovora* mutant strains [13]. Virulence assays using immature pears were conducted as previously described [109]. Briefly, immature pears were surface sterilized with 10% bleach, rinsed with sterile distilled water, and dried. Overnight bacterial cultures were adjusted to about 1 X 10^4 CFU/ml in 0.5X PBS, and 2 μl of this suspension was added to the surface of a pear and stab-inoculated with at maximum a 25 3/8 needle. The infected pears were incubated at 28°C and stored in a Tupperware container with plastic wrap to help create a better moisture lock under the lid. The humidity of the container and the pears was also maintained by covering the bottom of the container with sterile paper towel and wetting them every one to two days with sterile distilled water. The pears were photographed with a Red Imaging System (Alpha Innotech) and the extent of necrosis was manually quantified using ImageJ at 4, 5, 6, and 7 days post inoculation (dpi).

Apple shoot infection assays were conducted as previously described [48].

Table 2. Strains and plasmids used.		
Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
S17-λ*pir*		[26]
E. amylovora		
Ea1189	Wild type	[18]
Δ*ams*	Deletion of the *ams* operon	[110]
Δ*ecp1*	Deletion of AMY 2228, *ecp1::FRT*	This Study
Δ*ecp2*	Deletion of AMY 3311, *ecp1*::FRT	This Study
Δ*ecp3*	Deletion of AMY 3381, *ecp3*::FRT	This Study
Δ*gcp1*	Deletion of AMY 0335, *gcp1*::FRT	This Study
Δ*gcp2*	Deletion of AMY 0564, *gcp2*::FRT	This Study
Δ*gcp3*	Deletion of AMY 1504, *gcp3*::FRT	This Study
Δ*gcp4*	Deletion of AMY 2180, *gcp4*::FRT	This Study
Δ*gcp5*	Deletion of AMY 2435, *gcp5*::FRT	This Study
Plasmids		
pKD3	Contains CmR cassette and flanking FRT sites, CmR	[24]
pKD46	L-arabinose inducible lambda-red recombinase, ApR	[24]
pTL17	IPTG-inducible FLPase, KmR	[53]
pEVS141	pEVS143 with the CmR and GFP removed, vector control, KmR	[28]
pEVS143	Broad host range cloning vector, inducible CmR and GFP, KmR	[28]
pCMW75	*V. harveyi* DGC *qrgB,* over-expression vector, KmR	[102]
pCMW98	Active site mutant of pCMW75	[102]
pBRP2	*qrgB* over-expression vector, ApR	Pursley *et al*, unpub
pACE-*ecp1*	pEVS143 cmR::*ecp1*, over-expression vector, KmR	This Study
pACE-*ecp2*	pEVS143 cmR::*ecp2*, over-expression vector, KmR	This Study
pACE-*ecp3*	pEVS143 cmR::*ecp3*, over-expression vector, KmR	This Study
pACE-*gcp1*	pEVS143 cmR::*gcp1*, over-expression vector, KmR	This Study
pACE-*gcp2*	pEVS143 cmR::*gcp2*, over-expression vector, KmR	This Study
pACE-*gcp3*	pEVS143 cmR::*gcp3*, over-expression vector, KmR	This Study
pACE-*gcp4*	pEVS143 cmR::*gcp4*, over-expression vector, KmR	This Study
pACE-*gcp5*	pEVS143 cmR::*gcp5*, over-expression vector, KmR	This Study
pBBRlux	*V. harveyi* luciferase reporter vector, CmR	[52]
pACE-P*lsc*	pBBRlux::P*lsc*	This Study
pACE-P*ams*	pBBRlux::P*ams*	This Study

Results

Gene	ID	*E. Amylovora* PDEs and DGCs – Protein Domains	ORF Size (aa)
ecp1	AMY 2228	A	536
ecp2	AMY 3311		455
ecp3	AMY 3381		653
dcp1	AMY 0335		818
dcp2	AMY 0564		365
dcp3	AMY 1504		464
dcp4	AMY 2180		848
dcp5	AMY 2435		535



Gene	ID	*E. Amylovora* PDEs and DGCs – TM Domains	ORF Size (aa)
ecp1	AMY 2228	B	536
ecp2	AMY 3311		455
ecp3	AMY 3381		653
dcp1	AMY 0335		818
dcp2	AMY 0564		365
dcp3	AMY 1504		464
dcp4	AMY 2180		848
dcp5	AMY 2435		535

Fig. 1. The eight putative *Erwinia amylovora* c-di-GMP metabolizing enzymes. The EAL and GGDEF domains of these proteins are illustrated in blue and red, respectively, and are labeled

with the amino acid sequence present in the active site of each specific protein. Predicted protein domains are indicated in (A) while predicted transmembrane domains (TM) are indicated in (B) with gray bars. Domains were discovered using Pfam 25.0, and the TM domains were identified using TMHMM v. 2.0 [50]. All features of these cartoons, namely the amino acid sequence length of the TM and catalytic domains as well as the sequence of the protein itself, are drawn to scale. For interpretation of the references to color in this and all other figures, the reader is referred to the electronic version of this thesis.

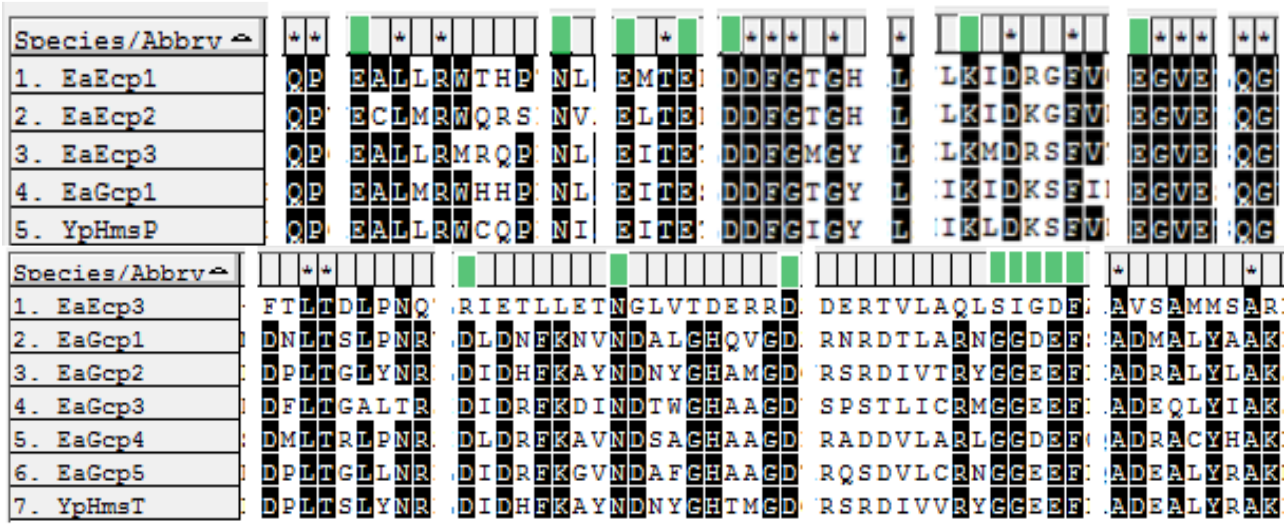


Fig. 2. Conservation of the key amino acid residues in the DGCs and PDEs of *E. amylovora.*

The EAL domain containing proteins from *Erwinia amylovora* are aligned compared with HmsP, an active PDE from *Yersinia pestis* (Top). The GGDEF-domain encoding proteins from *Erwinia amylovora* are aligned compared with HmsT, an active DGC from *Yersinia pestis* (bottom). Protein sequences were aligned using ClustalW on the MEGA 5.0 platform. Conserved amino acids (>80%) are highlighted in black. Residues required for enzymatic activity from this domain are indicated by the green bar above the amino acid alignments [66, 80]. The third amino acid

of a GGDEF-containing protein can retain function with either an aspartate or glutamate residue at the third amino acid position [80].

Erwinia amylovora encodes eight putative c-di-GMP metabolizing enzymes

Bioinformatic analysis of the *E. amylovora* genome revealed two genes encoding EAL domains, four genes encoding GGDEF domains, and two genes contained both domains (Fig. 1). No genes encoding an HD-GYP domain were identified. Each *E. amylovora* EAL domain encodes all of the critical amino acids required for catalysis, and thus could be potentially active PDEs (Fig. 2) [66]. Only one gene, *ecp3,* is missing critical amino acid residues required for catalysis, with a SIGDF sequence in place of GG(D/E)EF [80]. Thus, sequence analysis alone predicts that *E. amylovora* encodes five potentially active GGDEF domains and four potentially active EAL domains.

E. amylovora has two active PDEs and three active DGCs in the conditions examined

To determine if the enzymatic domains described above posses activity to synthesize or degrade c-di-GMP, we constructed over expression constructs of the eight genes on a plasmid vector under the control of the Ptac promoter [102]. Expression of these genes can then be induced with IPTG. The plasmids pCMW75 and pCMW98 were used as positive and negative controls, respectively [102]. The *qrgB* gene encodes a constitutively active cytoplasmic DGC from *V. harveyi* [101], and was cloned into the medium copy number and broad host range over-expression vector pEVS143 [28] to yield pCMW75 [102]. An active site mutant of *qrgB* (GGEEF to AAEEF) was made from pCMW75 to yield pCMW98. QrgB was chosen as a positive control because it has been shown to maintain DGC activity in several species including *V. cholerae* and *E. coli* [102], it contains no homolog in *Erwinia amylovora* so any phenotypes

associated with its over-expression should be c-di-GMP specific, and finally because we have the active site mutant already constructed, we can determine if any phenotypes regulated by *qrgB* are due to c-di-GMP synthesis. Wild type *E. amylovora* containing the vector control pEVS141 [28] was similarly examined.

Strains containing these over expression constructs were grown in LB with 1.0 mM IPTG and kanamycin in wild type *E. amylovora,* and the cells were harvested by centrifugation at mid-late log growth phase. The cell pellet was lysed with extraction buffer and the level of c-di-GMP in this cell extract in this buffer was quantified using HPLC-tandem MS-MS.

C-di-GMP was not observed in the wild-type strain containing the vector control, suggesting that the intracellular level of this molecule was below our limit of detection for *E. amylovora* grown in LB. Over-expression of *qrgB* resulted in relatively high levels of c-di-GMP compared to the vector control, and the level of c-di-GMP in strains over-expressing the active site mutant of *qrgB* was below detectable limits (Table 3). This result shows that c-di-GMP can be synthesized and detected in *E. amylovora.* Over-expression of *gcp1*, *gcp3*, or *gcp5* resulted in detectable levels of c-di-GMP, with *gcp3* producing the most c-di-GMP and *gcp5* producing the least. The level of c-di-GMP in strains over-expressing either *gcp2* or *gcp4* was below detection (Table 3).

As mentioned above c-di-GMP was not detected in the wild type strain, and, not surprisingly, we were not able to detect c-di-GMP in the strains over-expressing *ecp1*, *ecp2*, or *ecp3*. To assess the activity of these proteins we induced their expression in strains that also contained the plasmid pBRP2, a compatible plasmid encoding IPTG-inducible *qrgB* (Pursley, unpublished), such that the putative PDE was co-expressed with QrgB. Active PDEs were

identified by their ability to significantly reduce the intracellular level of c-di-GMP compared to the empty vector control (Fig. 3).

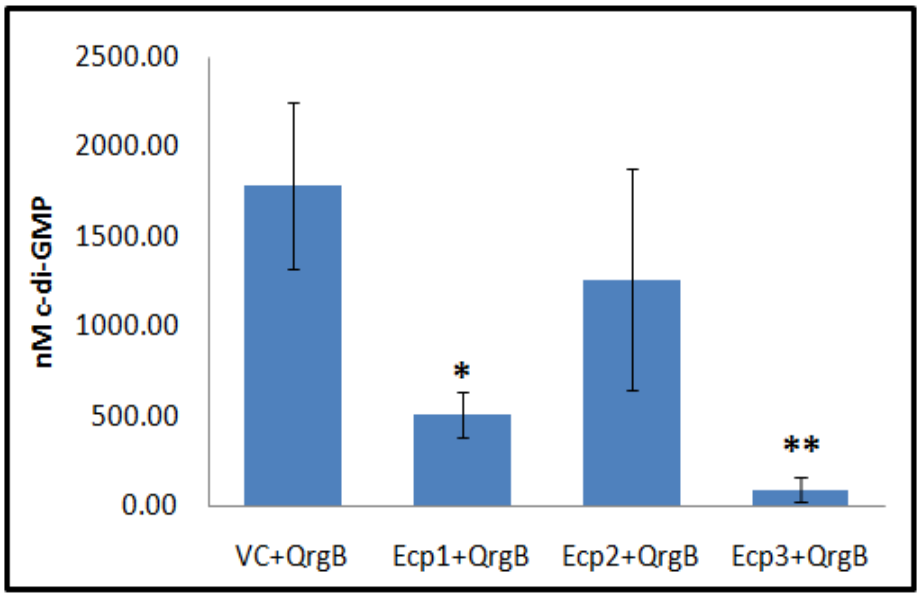


Fig. 3. Ecp1 and Ecp3 are active c-di-GMP PDEs. QrgB was co-over-expressed from pBRP2 with Ecp1, Ecp2, or Ecp3 in LB supplemented with kanamycin and 1.0 mM IPTG as described in the Materials and Methods section. Active PDE enzymes were identified from their ability to decrease the level of c-di-GMP in a strain with constitutively high c-di-GMP. A strain containing the empty vector was used as a control.

* indicates a p-value<0.05 compared to the VC+QrgB, as determined using the student's t-test; ** indicates p-value<0.05 compared to the Ecp1+QrgB.

As an alternative approach to determine which of these proteins are active in *E. amylovora,* in-frame unmarked deletion mutations of each gene were generated using lambda-red mediated high frequency recombination [24]. Metabolites were extracted from each of these strains and the levels of c-di-GMP were determined using HPLC-MS-MS. The observation that c-di-GMP was not detected in any of the DGC mutant strains is expected because the c-di-

GMP level of the wild-type strain was below detectable limits (data not shown). Of the PDE mutants, we were only able to measure an increased level of c-di-GMP in the *⊿ecp3* mutant strain (Table 2). This observation correlates with our finding that over-expression of *ecp3* most strongly reduced c-di-GMP production from *qrgB.*

Therefore, measuring the intracellular levels of c-di-GMP when each GCP and ECP was over-expressed or mutated revealed that Ecp1 and Ecp3 are the two active PDEs in *E. amylovora* and that Gcp1, 3, and 5 are the active DGCs in *E. amylovora*.

Table 3. Intracellular c-di-GMP (nM)	
Vector Control	ND
QrgB	1050 ± 248
QrgB*	ND
Gcp1	76.6 ± 3.39
Gcp2	ND
Gcp3	176 ± 71.8
Gcp4	ND
Gcp5**	4.62 ± 0.420
*⊿ecp3***	34.5 ± 13.8

*Active site mutant of QrgB

**15 mL culture volume compared to 5 mL for the other strains.

C-di-GMP negatively regulates swimming motility in *E. amylovora*

Swimming motility in most plant pathogenic bacteria including *E. amylovora* is facilitated by the helical rotation of between two to seven flagella. This motility is not observed within the host, though it has been shown that plant pathogenic bacteria display a unique chemotaxis for dicarboxylic acid, a component found in apple nectar [67, 68]. It has also been shown that c-di-GMP generally represses flagellar motility [45, 57, 71, 88, 106]. To determine which c-di-GMP synthesis and degradation enzyme from *E. amylovora* can regulate swimming motility, we stab-inoculated strains over-expressing each *Erwinia amylovora* DGC and PDE, as

well as our controls, into low-density LB agar plates amended with kanamycin and 1.0 mM IPTG. We observed no change in swimming motility in strains over-expressing *ecp1*, *ecp2*, or *ecp3* (data not shown). This observation is not surprising as the levels of c-di-GMP in the wild type strain grown in LB are already low as described above. Strains over-expressing gcp*3* or *gcp5* were essentially non-motile (Fig. 4A, C), suggesting that c-di-GMP production from these enzymes inhibited motility. However, over-expression of *gcp1* did not result in a motility phenotype, even though over-expression of this enzyme in liquid media increased the intracellular concentration of c-di-GMP (Fig. 4A).

We also examined the motility of the DGC and PDE mutants. Surprisingly, the *⊿ecp3* mutant strain exhibited increased swimming motility compared to the wild type (Fig. 4B, D). This observation was surprising as deletion of this gene increases the intracellular concentration of c-di-GMP. *⊿gcp2* was slightly deficient in motility compared to the wild-type (Fig. 4B, D) but over-expression of this gene did not result in any measurable phenotypes (Fig. 4A). As over-expression of *gcp3* or *gcp5* reduced motility, we predicted that mutation of these genes would increase motility. However, swimming motility was not affected in strains lacking *gcp3* or *gcp5* (Fig. 4B). We hypothesize this may be due to two factors. First, we do not know if *gcp3* and *gcp5* are normally expressed in the conditions examined. If their basal expression is low, mutation of these genes would not result in a measureable phenotype. Secondly, it has been shown that DGCs can be redundant [90]. Therefore, we predict a *⊿gcp3 ⊿gcp5* double mutant may be hyper motile. These results show that flagellar motility in *E. amylovora* is generally repressed by c-di-GMP.

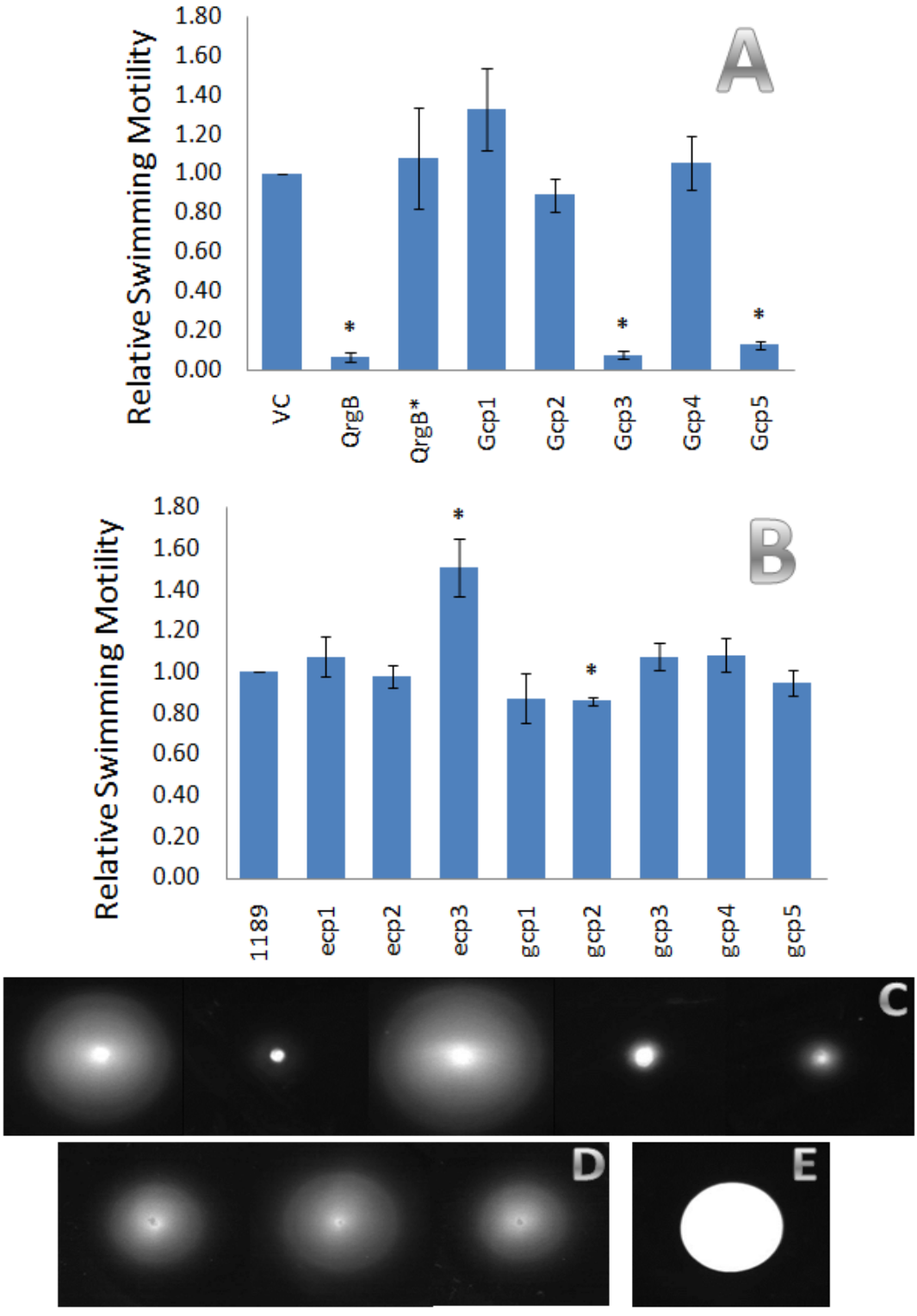


Fig. 4. C-di-GMP alters motility in *E. amylovora*. A) Motility was examined upon over-expression of the proteins indicated in low-density motility agar supplemented with 1.0 mM

IPTG and kanamycin. B) Motility of the mutants was similarly examined C) Images of the motility of strains containing (C) left to right, the vector control or over-expressing QrgB, QrgB (AAEEF), Gcp3, and Gcp5. (D) left to right, wild type, *Δecp3, Δgcp2*. Overnight cultures were stab-inoculated into 0.3% LB agar plates in triplicate and incubated at 28°C for exactly 20 hours. Motility area was pixilated using ImageJ and normalized to a 1 cm^2 sticker (E) as an area control on each plate. Values were again normalized to the value of pEVS141, the vector control or to the wild type. Data shown represent the average of three technical replicates for each of three biological replicates.

* indicates a p-value<0.05, determined using the student's t-test.

C-di-GMP positively regulates amylovoran production

C-di-GMP regulates EPS production in *E. coli*, *Y. pestis*, *P. aeruginosa*, and other prokaryotes [12, 45, 57, 93]. To determine if c-di-GMP similarly regulates EPS production in *E. amylovora*, we inoculated our mutant strains into MBMA medium and quantified the amount of amylovoran in the culture supernatants using the cetylpyridinium chloride (CPC) binding assay [7]. CPC binds to anionic polysaccharides and precipitates them, allowing for the correlation of the turbidity of the supernatant with the amount of amylovoran secreted [84]. The *Δams* strain was used as a negative control because it does not produce amylovoran [110]. Normal MBMA was used for analysis of amylovoran production in the mutant strains. Of these, only the *Δecp3* strain showed increased amylovoran secretion compared to the wild type strain (Fig. 5B). As expected, the *Δams* strain was shown to be deficient in amylovoran production in this experiment (Fig. 5B).

Growth of the over-expression strains in normal MBMA medium led to autoaggregation at the bottom of the tube in strains that produce high levels of c-di-GMP (Fig. 8), resulting in inconsistent data. The culture density of the high c-di-GMP strains was lower than the culture density of the low c-di-GMP strains (data not shown), and we suspected that increased c-di-GMP led to nutrient limitation. This issue was circumvented by growing our over-expression strains in a mixture of one part LB to three parts MBMA called liquid gold (LG). Over-expression of *qrgB* resulted in a three-fold increase of amylovoran production when compared to the vector control. Additionally, the level of amylovoran secreted in a strain over-expressing the active site mutant of *qrgB* was similar to that of the vector control (Fig. 5A), indicating that this method is suitable for correlating amylovoran production with the level c-di-GMP. Strains over-expressing *dgc3* and *dgc5* secrete between two- and three-fold more amylovoran than the vector control (Fig. 5A). These results demonstrate that amylovoran production in *E. amylovora* is positively regulated by c-di-GMP in these conditions.

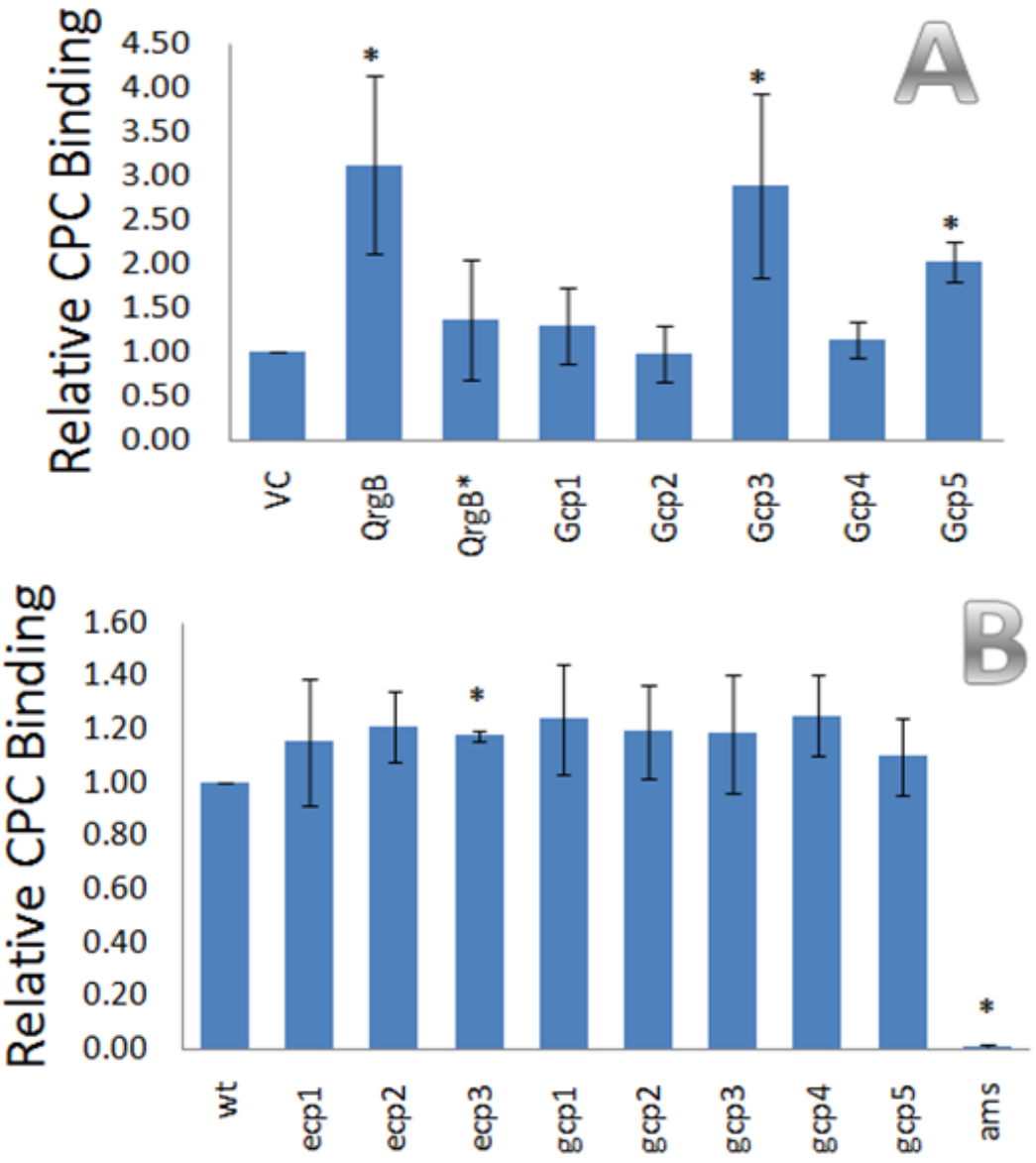


Fig. 5. C-di-GMP increases secretion of amylovoran. A) Over-expression strains were grown in LG supplemented with kanamycin and 0.1 mM IPTG. B) Wild type and mutant strains were grown in MBMA. Amylovoran was measured as described in the Materials and Methods section. The values shown were normalized to the value of the vector control (pEVS141, A) or the wild type (B). The *ams* mutant is deficient in amylovoran production, and was used as a negative control.

* indicates a p-value<0.05, determined using the student's t-test

EPS production was also estimated using the CR binding assay. EPS production is directly correlated to the binding of these chemicals, and is indicated by the degree of red pigmentation (reviewed in [70]). Colonies of strains over-expressing *qrgB*, *gcp3*, and *gcp5* had greater binding of these dyes than the vector control and the *qrgB** controls (Fig. 6). It was also noted that over-expression of *gcp1* did not result in increased binding compared to the controls. Binding of these dyes was also increased in the *Δecp3* mutant strain when compared to the base-line levels of binding observed in the wild-type (data not shown). These results further indicate that c-di-GMP positively regulates EPS production in these conditions.

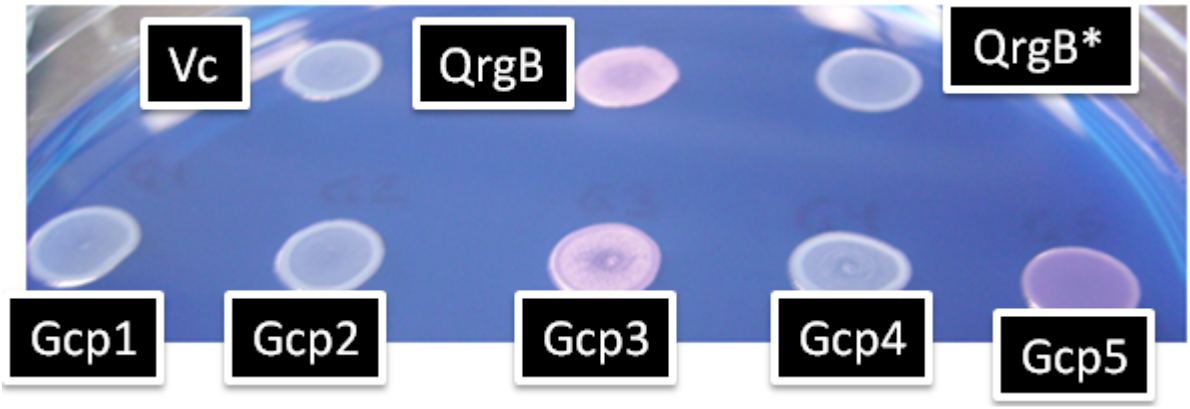


Fig. 6. C-di-GMP induces EPS-dependent binding of CR. Over-expression strains of *E. amylovora* were added to LB agar plates supplemented with kanamycin, 0.1 mM IPTG, and CR. CR binding was assayed visually.

C-di-GMP positively regulates transcription from the *ams* promoter

To determine if increased amylovoran secretion in strains with elevated c-di-GMP is due to transcriptional regulation, we fused the putative promoter region from -501 to -1 of the 15.8 kb *ams* operon upstream of the *luxCDABE* reporter genes of the pBBR-*lux* reporter vector [52].

Over-expression of *qrgB* resulted in three-fold induction of transcription from the promoter of the *ams* operon when compared to the vector control, while transcription in a strain over-expressing the active site mutant of *qrgB* was not greater than that of the vector control (Fig. 7). Over-expression of *dgc3* and *dgc5* resulted in 3- and 1.5-fold higher transcription from the promoter of the *ams* operon compared to the vector control, respectively (Fig. 7). However, a *cis* element at -578 to -501 called the FA site that is implicated in RcsB-mediated transcriptional regulation of this operon was not included in the fusion construct [47]. We were surprised to have measured transcription regulation from this promoter without the binding site of a known transcriptional activator and future studies should examine if this element plays a role in c-di-GMP regulation of this promoter. These results show that in these conditions, c-di-GMP is a positive transcriptional regulator of the *ams* operon in *E. amylovora*.

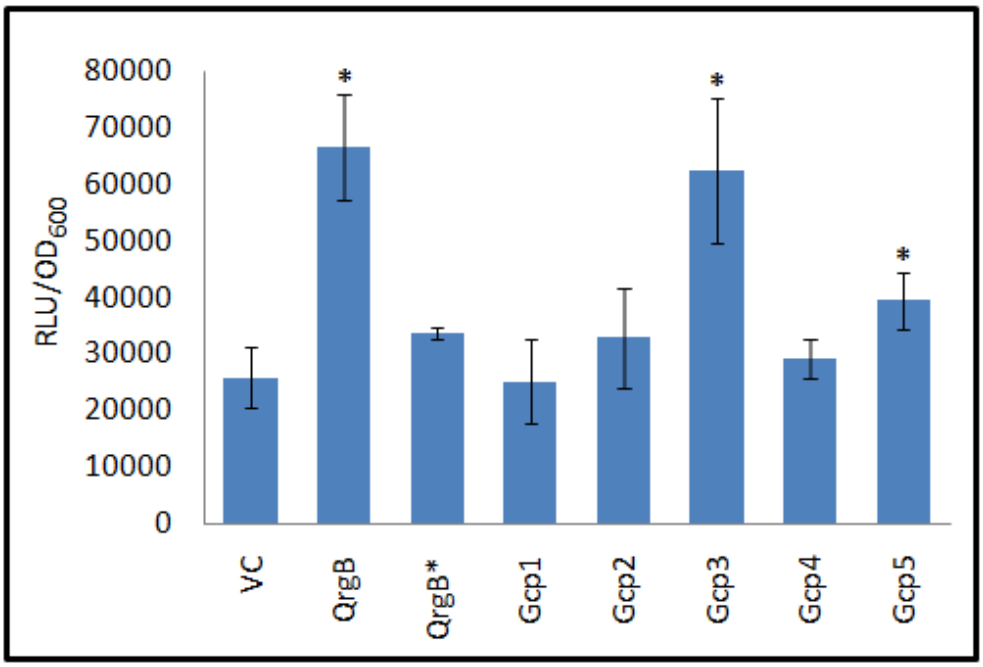


Fig. 7. C-di-GMP induces expression of the *ams* operon. Cultures were grown overnight in 150 µL LB supplemented with chloramphenicol and kanamycin in a 96 well plate with agitation at

28°C and then transferred to a new 96 well plate with a 96-pin replicator tool (V&P Scientific) into 150 μL LB supplemented with chloramphenicol, kanamycin, and 0.1 mM IPTG. Plates were grown at 28°C with agitation, and maximum luminescence was recorded using a SpectraMax M2 multimode microplate reader at eight hours and normalized to the culture OD_{600}. These data represent the mean of three biological and three technical replicates.

* indicates a p-value<0.05, determined using the student's t-test

C-di-GMP induces autoaggregation

Autoaggregation is a biofilm-associated phenotype used to describe bacteria of the same species that are attached or clumped together. This trait is a survival mechanism, much like biofilm formation, and autoaggregation has been linked to c-di-GMP levels in several species [23, 38, 45, 73]. Cell aggregates were observed on the bottom of each tube in strains over-expressing *dgc3* and *dgc5*, as well as the *qrgB* positive control, while the cultures with low c-di-GMP levels are of uniform turbidity (Fig. 8), suggesting c-di-GMP induces autoaggregation in *E. amylovora*.

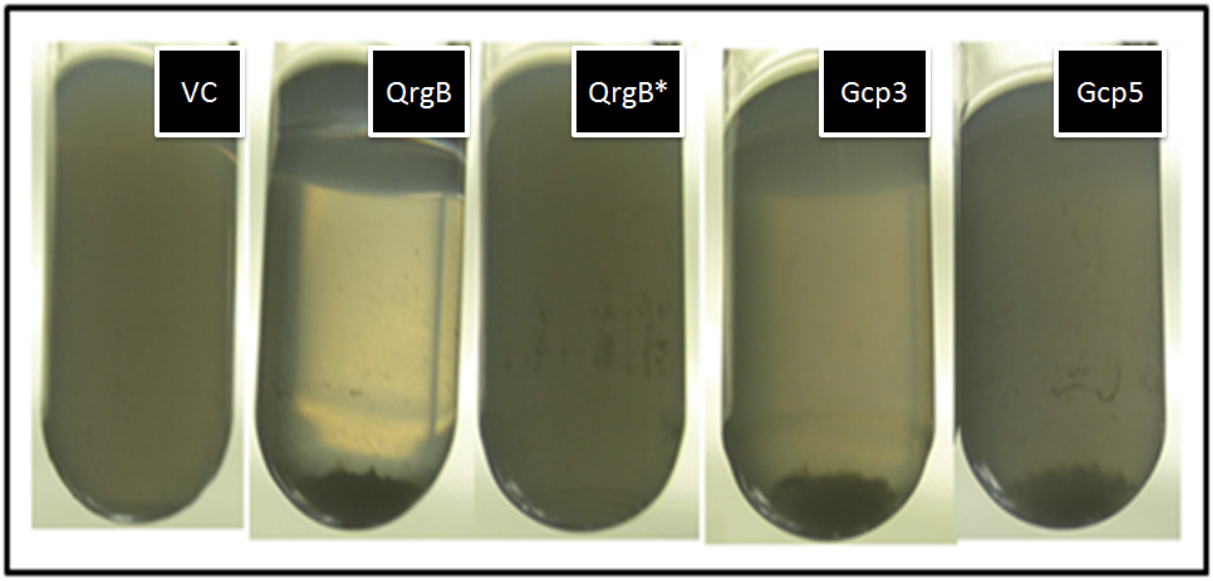


Fig. 8. C-di-GMP induces autoaggregation in *E. amylovora*. Overnight cultures of strains containing the over-expression vectors grown in LB with kanamycin were added to five mL MBMA medium supplemented with kanamycin and 0.1 mM IPTG (in a 18 by 150 mm glass tube) to an initial OD_{600} of 0.1. Cultures were incubated with agitation at 28°C. Aggregation was qualified by visualization after 24 hours. From left to right: vector control, QrgB, QrgB* (AAEEF), Gcp3, Gcp5. All strains that have low levels of c-di-GMP resemble the vector control and QrgB* cultures (not shown).

C-di-GMP positively regulates biofilm formation

Amylovoran production and biofilm formation are important virulence factors during infection and colonization of susceptible host tissues by *E. amylovora* [48, 110] and amylovoran production is required for biofilm formation *in vitro* and *in vivo* for this strain [48]. Our results thus far have shown that increased c-di-GMP in *E. amylovora* induces amylovoran production and autoaggregation. To further examine the role of c-di-GMP in biofilm formation, we examined the impact of over-expression and mutation of each DGC and PDE using a modified

static biofilm assay (Fig. 9). Similar to our results for amylovoran production and autoaggregation, over-expression of *qrgB*, *gcp3*, and *gcp5* resulted in four-, six-, and five-fold respective increases in biofilm formation over the vector control. Over-expression of the active site mutant of *qrgB* resulted in biofilm formation similar to that of the vector control. Analysis of the mutant strains revealed that biofilm formation in the *Δecp3* mutant strain was about four fold higher than that of the wild type. No other mutants displayed significantly different levels of biofilm formation.

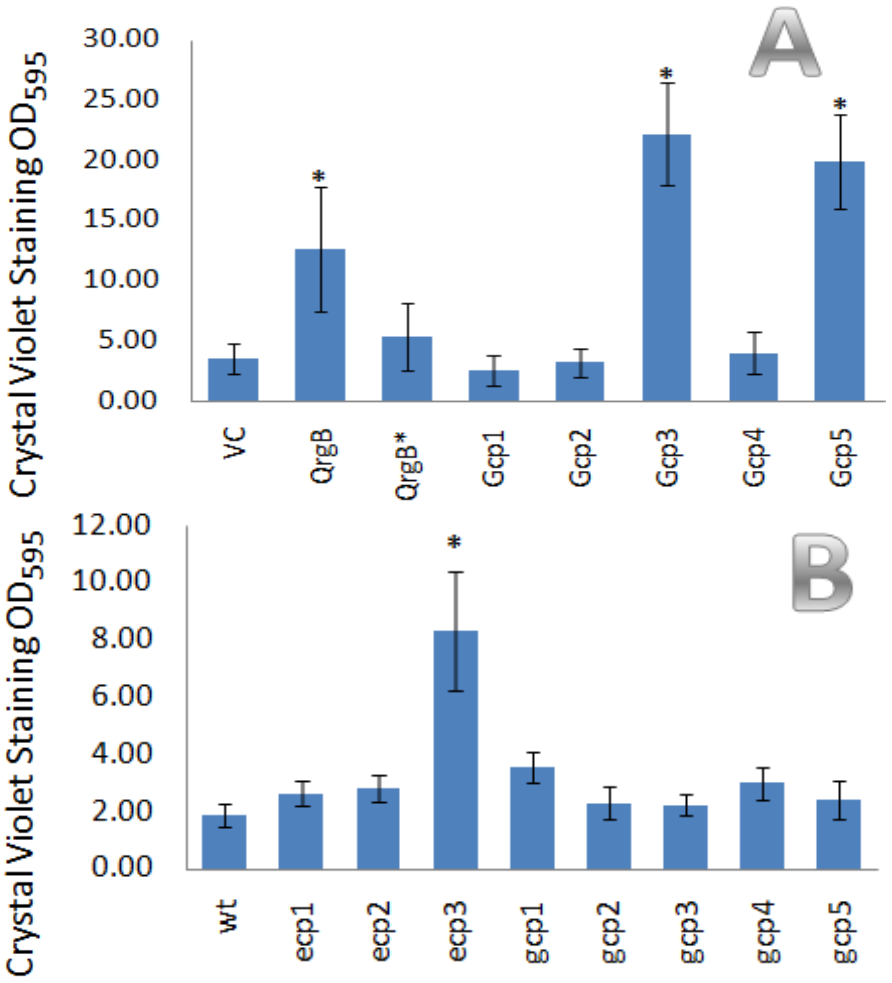


Fig. 9. C-di-GMP induces biofilm formation in static conditions. A) Over-expression strains were grown in LG media supplemented with kanamycin and 1.0 mM IPTG and biofilm formation

was determined by quantifying crystal violet binding as described. B) Wild type and mutant strains grown in LG media in a 48-well plate.

* indicates a p-value<0.05, determined using the student's t-test.

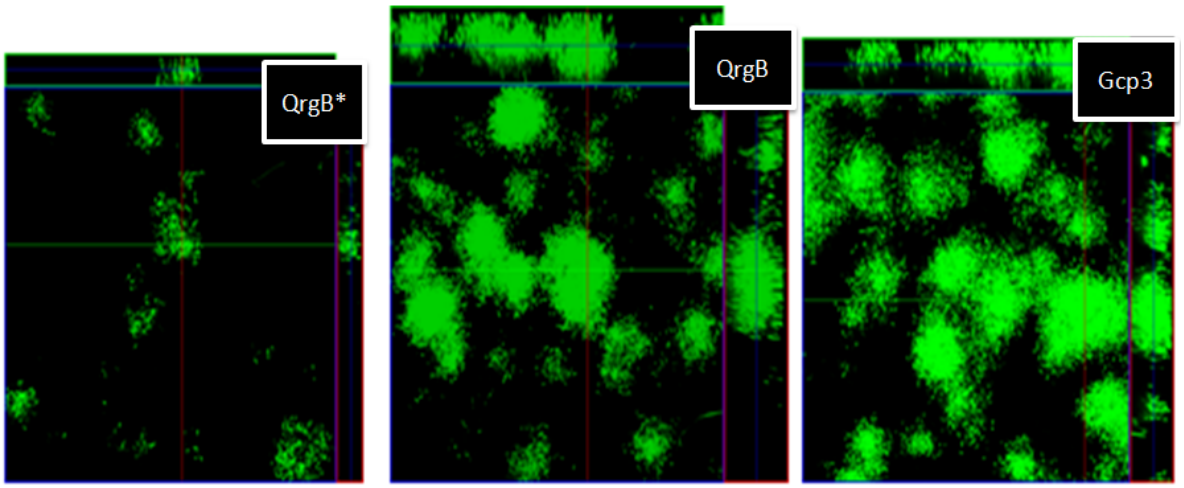


Fig. 10. C-di-GMP induces biofilm formation in flowing conditions. MBMA medium supplemented with 0.1 mM IPTG and kanamycin was pumped through the chamber at 12 mL/hr at room temperature for two days. Random fields were analyzed using confocal microscopy to qualify the biofilms.

To further this examination, biofilm formation of *E. amylovora* was determined using a flow cell system. The flow cell apparatus is designed to more accurately portray biofilm formation in steady state nutrient availability, and the results are then analyzed by confocal laser scanning microscopy. Before fresh media is continuously pumped over the flow cell chamber, cell cultures are introduced and allowed one hour to allow for initial surface attachment. As fresh media is then passed continuously through the chamber, the planktonic cells are gently washed away while the attached cells remain to mature into a biofilm macrocolony. The mature biofilm is rooted to the surface by the attached cells and grows as

the bacteria multiply and excrete EPS (Reviewed in [39]). Similar to the results of our static assays, biofilm formation was highly induced in flowing conditions when *qrgB* and *gcp3* were over-expressed in comparison to biofilm formation of a strain over-expressing *qrgB** (Fig. 10). For completeness, we will test the Gcp5 over-expression strain and the *⊿ecp3* mutant strain in flowing conditions. These results show that biofilm formation by *E. amylovora* is positively regulated by c-di-GMP in these conditions.

C-di-GMP represses virulence in *E. amylovora* apple shoot infection

Amylovoran production/biofilm formation and the secretion of avirulence and effector proteins via the T3SS are important in the virulence of *E. amylovora* [1, 48, 98, 110]. These factors have been linked to c-di-GMP in several plant pathogenic bacteria including *X. campestris*, *D. dadantii*, and *X. fastidiosa* [19, 78, 79, 108]. To examine the role of c-di-GMP in of *E. amylovora* apple shoot infection, we introduced the *⊿ecp3* mutant strain into the leaf tip of an apple tree scion. The infection profile of this strain was deduced using the wild type strain and the *⊿ams* mutant strain as positive and negative controls, respectively. The wild-type strain exhibited robust vascular infection indicated by wilting, necrosis, and the presence of bacterial ooze, accompanied by migration into neighboring shoots. The *⊿ams* mutant strain was not virulent. Infection of the *⊿ecp3* mutant strain, which contains an elevated level of c-di-GMP (Table 2), resulted in necrosis of the immediate area of inoculation but was not capable of migration within the host. Additionally, this mutant was also not confined to the xylem (Fig. 11). Though time permitted this experiment to be run only once, it must be repeated to confirm this result. However, this preliminary result indicates that c-di-GMP inhibits

pathogenesis of *E. amylovora* in the host by blocking the vascular migration of these bacteria within the host.

Fig. 11. C-di-GMP inhibits virulence and migration within the host. Left – wild type. Notice the characteristic ooze seeping out of the shoot in the box. The infection was able to spread to neighboring host tissues as indicated by the necrosed tissues on the leaves at the top. Right - *Δecp3* is unable to spread through the vascular tissue of the shoot. Overnight cultures were grown in LB and diluted in PBS. Sterile scissors were dipped into the diluted culture and used to clip the tip of a young leaf. Infection was recorded at seven and 14 dpi. The images above represent disease at 14 dpi.

C-di-GMP in regard to virulence in the immature pear infection assay

In addition to the apple shoot infection assays for virulence, immature pear infection assays were also conducted. The results indicated that disease progression in the *Δecp1* and *Δgcp1* mutant strains was statistically lower than that of the wild type strain, while that of *Δgcp4* was statistically higher. The *Δams* mutant strain did not cause disease (Fig. 12). The mutant strains must be complemented to confirm the virulence phenotypes.

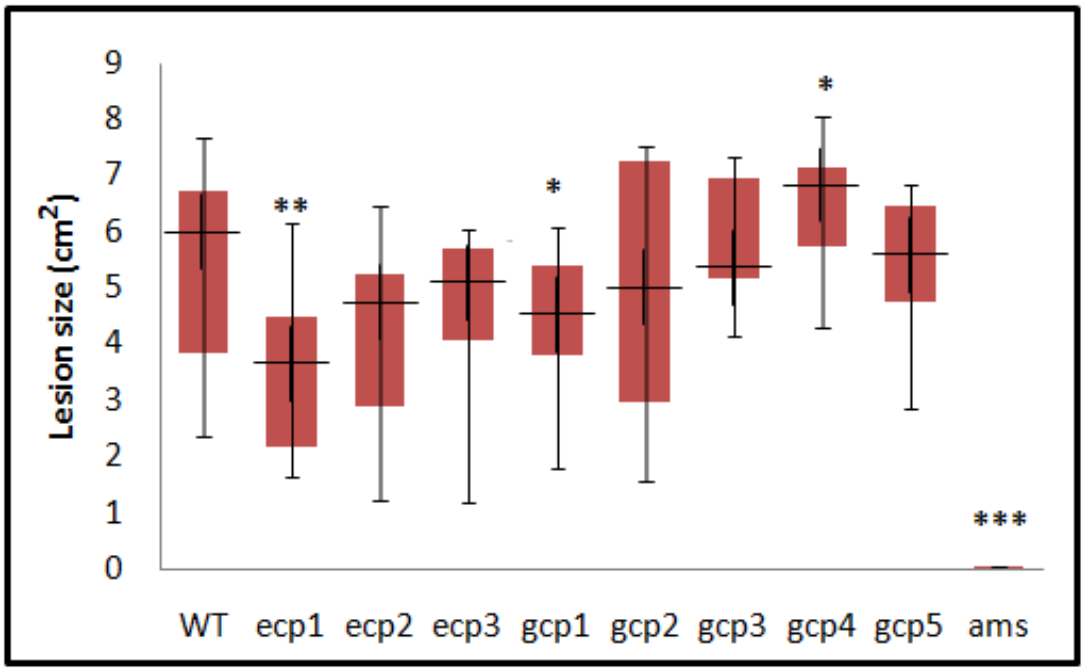


Fig. 12. C-di-GMP relevance to immature pear infection. Immature pears were rinsed in tap water, surface sterilized in 10% bleach, and then soaked in sterile RO water and allowed to dry. Overnight cultures were grown in LB and diluted to an OD_{600} of about 0.002. 2 μL of dilute culture (about 10^4 CFU) was added to the surface of each pear and introduced into the fruit using a 25 3/8 gauge hypodermic needle. Infected pears were stored at 28°C and lesion size was recorded at four, five, and six dpi. These data represent lesion size at 6 dpi.

*represents $p < 0.2$; ** represents $p < 0.05$; *** represents $p < 0.001$

DISCUSSION

C-di-GMP metabolism in *E. amylovora*

In this study, we defined the catalytically active DGCs and PDEs in *E. amylovora*. There are six ORFs encoding a conserved GGDEF domain, and of these, *gcp1*, *gcp3*, and *gcp5* were shown to enhance the intracellular concentration of c-di-GMP, with *gcp3* the most capable of increasing the level of c-di-GMP and *gcp5* the least. The ORF annotated as *ecp3* encodes a degenerate GGDEF catalytic domain, SIGDF, and was thus not predicted to have DGC activity [80]. The ORFs annotated as *gcp2* and *gcp4*, however, were predicted to be active DGCs based on *in silica* data [80], but were shown to have no such activity. There was no detectable c-di-GMP in any of the GGDEF mutant strains. In *S. enterica*, deletion of individual DGCs was ineffectual while deletion of multiple DGCs resulted in a measurable phenotype, indicating a redundancy among DGCs [90]. Therefore we predict that deletion of two or of all three active *E. amylovora* DGCs may be required for a DGC mutant phenotype.

There are also four ORFs encoding a conserved EAL domain, and of these, *ecp1* and *ecp3* were capable of degrading intracellular c-di-GMP. The ORFs encoding *ecp2* and *gcp1* did not demonstrate this ability, despite encoding all amino acids required for PDE activity [66]. Of the EAL mutant strains, only the *Δecp3* mutant strain displayed a measurable increase of c-di-GMP in these conditions, indicating that *ecp3* is the major *E. amylovora* PDE.

The inactivity of *gcp2* is surprising because it is a homolog of *hmsT*, which encodes the main DGC of *Y. pestis* [12]. In fact of the 10 GGDEF or EAL domains included in this study, only one encodes a degenerate sequence. One possible explanation for the apparent lack of c-di-GMP metabolism by these proteins is that they require an environmental signal or post-

translational modification for activation. Moreover, we have not examined the native expression levels of these genes. Alternatively, some of these over-expressed proteins may not be accumulating in the cell, or they may serve a function apart from c-di-GMP signaling.

C-di-GMP signaling in *E. amylovora*

In *E. amylovora*, sessile behaviors such as biofilm formation and aggregation are dependent on amylovoran production, which is also required for disease [48, 110]. In this study, we've shown that c-di-GMP is a positive regulator of amylovoran production (Figs. 5-7), aggregation (Fig. 8), and biofilm formation (Figs. 9, 10) in *E. amylovora*, and that swimming motility is negatively regulated (Fig. 4).

The first exception to these conclusions involves the results from the swimming motility assay of two mutant strains: *Δecp3* and to a lesser extent, *Δgcp2*. In *E. amylovora*, *gcp2* encodes a non-functional DGC as indicated by the tandem HPLC-MS-MS data (Table 2) and all other studies involving the over-expression of or the mutation of this ORF did not result in a result different from the controls. Furthermore, mutation of an active DGC should result in increased flagellar motility, yet, mutation of *gcp2* led to decreased motility. The motility deficiency of this mutant remains a puzzle. Ecp3 is the major PDE of *E. amylovora* and the *Δecp3* mutant strain has an elevated level of intracellular c-di-GMP, so one should expect that flagellar motility in this mutant would be impeded. The observed increase in swimming motility of this strain is also perplexing. Our evidence suggests that c-di-GMP does negatively regulate flagellar motility in *E. amylovora;* yet, *gcp2* and *ecp3* appear to impact motility independently of alterations to global c-di-GMP pools.

Also puzzling is our observation that Gcp1 contains an active GGDEF and EAL domain as determined *in silico*, and over-expression of this gene results in an increase of intracellular c-di-GMP, but we have yet to determine a phenotype as a result of that increase. Bioinformatical analysis of this ORF has revealed that it is: 1) very well conserved among a diverse group of bacteria 2) 300 bp upstream of the gluconate-2 dehydrogenase operon and 3) homologous to proteins with both DGC activity and adenylate cyclase activity. There are also three PAS domains which hint at oxygen-sensing capabilities much like the *E. coli* Dos protein which has two PAS domains as well as both a GGDEF and EAL motif. The *E. coli* Dos protein was also thought to be an active adenylate cyclase [82], so Gcp1 might function similarly. Proteins encoding both a GGDEF and EAL domain have been shown to possess both c-di-GMP synthesis and degradation activity. The *scrC* gene from *V. parahemolyticus* encodes active EAL and GGDEF domains, and in the absence of the ScrAB proteins, has intrinsic DGC activity, though with those proteins it is an active PDE [31]. *gcp1*, however, is apparently not in an operon, though its proximity to the glutamate-2-dehydrogenase genes should not be overlooked. Regardless, understanding why this protein is capable of increasing the intracellular concentration of c-di-GMP without altering any of the phenotypes associated with a high c-di-GMP state is a research question requiring further examination One possibility is that Dgc1 is sequestering c-di-GMP through binding of c-di-GMP dimers to its RxxD allosteric inhibition site. This site, also called the I-site, is a conserved four amino acid sequence located five amino acids away from the GGDEF motif, and has been identified as the core c-di-GMP binding site. This site is conserved among GGDEF domains, and is hypothesized to be involved in c-di-GMP regulation via product inhibition [20]. Thus, the increased c-di-GMP observed upon over-

expression may not be freely available to regulate downstream phenotypes. This hypothesis could be testing by mutating those residues such that Gcp1 no longer binds c-di-GMP at its RxxD site.

C-di-GMP signaling and disease in *E. amylovora*

Our preliminary results have also indicated that c-di-GMP is an important regulator of disease in this pathogen, as an altered disease phenotype of the PDE mutant strain *Δecp3* was observed in the apple shoot infection assay (Fig. 11). This strain was able to cause necrosis of the host tissue, suggesting that *Δecp3* utilizes the T3SS for translocation of effector molecules, and we predict that electron microscopy of these infected tissues will reveal biofilm formation and amylovoran production. The observation that the disease caused by this strain was restricted to the first few centimeters of host tissues and was not limited to the vascular tissue of the host suggests that this strain is not capable of the xylem migration necessary for metastasis of the disease.

In contrast to the wild type strain, the *Δecp3* mutant strain has an increased level of c-di-GMP (Table 1), produces more amylovoran, is hyper-aggregative, and produces more biofilms. Though it is interesting that this strain is hyper-motile in regard to flagellar motility, this characteristic is likely irrelevant to a disease model because *E. amylovora* does not exhibit flagellar motility within the host and likely relies on Type IV pilus-mediated twitching motility [68]. Several PDE deficient plant pathogenic bacteria also show similar virulence phenotypes. In *X. fastidiosa* and in *X. campestris*, mutant strains with hyper-aggregative hyper-biofilm forming phenotypes are similarly unable to spread within the host and cause systemic infection in their respective plant host strains [19, 78]. Additionally, the HmsP deficient mutant of *Y.*

pestis has elevated c-di-GMP levels and is similarly unable to cause systemic disease in a mouse model [12]. We propose that the *Δecp3* mutant strain of *E. amylovora* is unable to spread within the host because it is likely compromised in regard to twitching motility and/or unable to disperse from the mature biofilm. Future research studying c-di-GMP in *E. amylovora* should further explore the role of c-di-GMP in disease and determine which of the *gcp* and *ecp* enzymes are active *in planta*. My research described here provides a solid foundation to continue to explore this question.

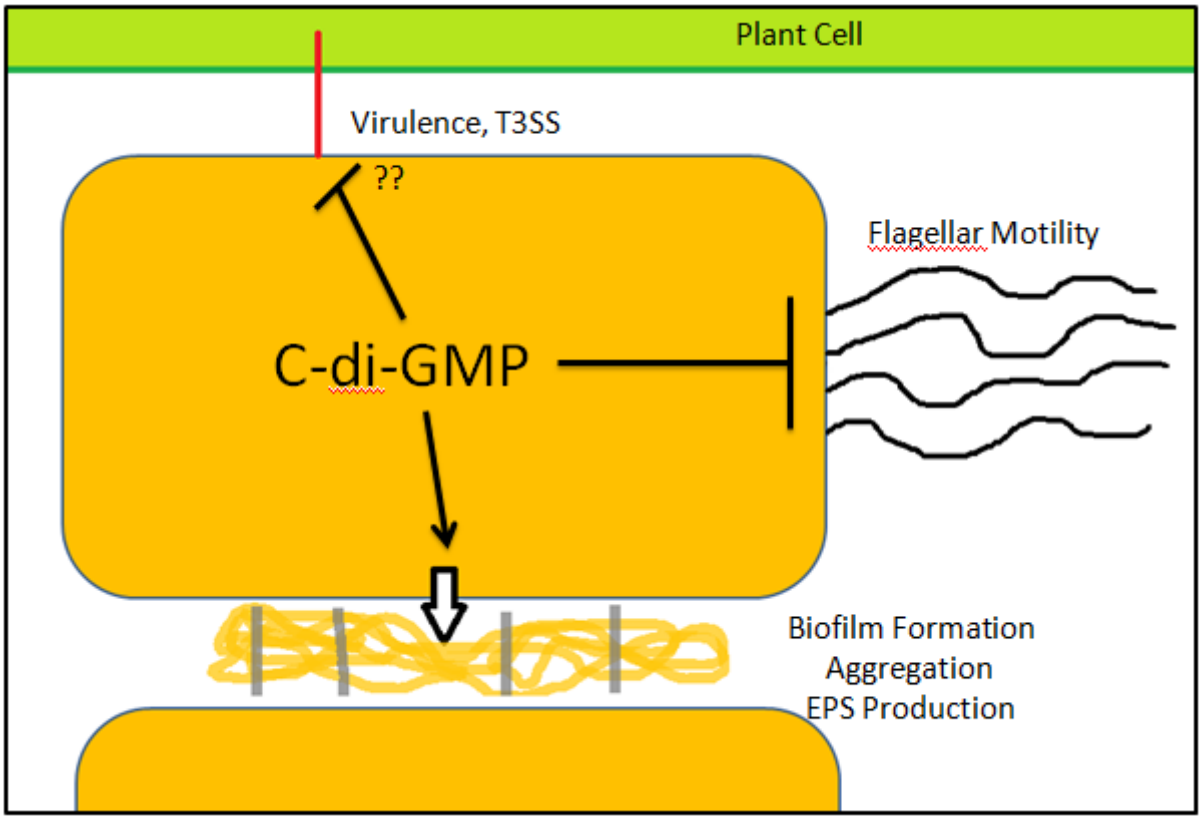


Fig. 13. Summary model of c-di-GMP regulation in *E. amylovora*. Our results indicate that in *E. amylovora*, c-di-GMP promotes biofilm formation, aggregation, and EPS production while negatively regulating flagellar motility. Preliminary data suggests that c-di-GMP might also inhibit virulence in apple trees.

REFERENCES

REFERENCES

1. Alfano, J.R. and A. Collmer. The type III (hrp) secretion pathway of plant pathogenic bacteria: Trafficking harpins, avr proteins, and death. Journal of Bacteriology, 1997. **179**(18): p. 5655-5662.

2. Aloni, Y., R. Cohen, M. Benziman, and D. Delmer. Solubilization of the UDP-glucose:1,4-β-D-Glucan 4-β-D-Glucosyltransferase (Cellulose Synthase) from *Acetobacter xylinum.* The Journal of Biological Chemistry, 1983. **258**(7): p. 4419-4423.

3. Ayers, A.R., S.B. Ayers, and R.N. Goodman. Extracellular polysaccharide of *Erwinia amylovora*: A correlation with virulence. Applied and Environmental Microbiology, 1979. **38**(4): p. 659-666.

4. Bachmann, F.M. The migration of *Bacillus amylovorus* in the host tissues. Phytopathology, 1913. **3**: p. 3-14.

5. Bailey, T.L. and M. Gribskov. Combining evidence using p-values: Application to sequence homology searches. Bioinformatics, 1998. **14**(1): p. 48-54.

6. Baker, K.F. Fire blight of pome fruits: The genesis of the concept that bacteria can be pathogenic to plants. Hilgardia, 1971. **40**: p. 603-633.

7. Bellemann, P., S. Bereswill, S. Berger, and K. Geider. Visualization of capsule formation by *Erwinia amylovora* and assays to determine amylovoran synthesis. International Journal of Biological Macromolecules, 1994. **16**: p. 290-296.

8. Bennett, R.A. and E. Billing. Origin of the polysaccharide component of ooze from plants infected with *Erwinia amylovova.* Journal of General Microbiology, 1980(116): p. 341-349.

9. Berg, H.C. How bacteria swim. Scientific American, 1975(233): p. 36-44.

10. Bernhard, F., D.L. Coplin, and K. Geider. A gene cluster for amylovoran synthesis in *Erwinia amylovora*: Characterization and relationship to *cps* genes in *Erwinia stewartii.* Molecular and General Genetics, 1993(239): p. 158-168.

11. Bobrov, A.G., O. Kirillina, S. Forman, D. Mack, and R.D. Perry. Insights into *Yersinia pestis* biofilm development: Topology and co-interaction of Hms inner membrane proteins involved in biofilm exopolysaccharide production. Environmental Microbiology, 2008. **10**: p. 1419-1432.

12. Bobrov, A.G., O. Kirillina, D.A. Ryjenkov, C.M. Waters, P.A. Price, J.D. Fetherston, D. Mack, W.E. Goldman, M. Gomelsky, and R.D. Perry. Systematic analysis of cyclic di-GMP

signalling enzymes and their role in biofilm formation and virulence in *Yersinia pestis.* Molecular Microbiology, 2011. **79**(2): p. 533-51.

13. Bogdanove, A.J., D.W. Bauer, and S.V. Beer. *Erwinia amylovora* secretes DspE, a pathogenicity factor and functional AvrE homolog, through the Hrp (type III secretion) pathway. Journal of Bacteriology, 1998. **180**(8): p. 2244-2247.

14. Bordeleau, E., L.C. Fortier, F. Malouin, and V. Burrus. C-di-GMP turn-over in *Clostridium difficile* is controlled by a plethora of diguanylate cyclases and phosphodiesterases. PLoS Genetics, 2011. **7**(3): p. e1002039.

15. Boureau, T., H. ElMaarouf-Bouteau, A. Garnier, M.N. Brisset, C. Perino, I. Pucheu, and M.A. Barny. DspA/E, a type III effector essential for *Erwinia amylovora* pathogenicity and growth in planta, induces cell death in host apple and nonhost tobacco plants. Molecular Plant-Microbe Interactions, 2006. **19**(1): p. 16-24.

16. Burrill, T.J. Pear blight. Transactions of the Illinois State Horticultural Society, 1877. **11**(New Series): p. 114-116.

17. Burrill, T.J. New species of *Micrococcus* (bacteria). American Naturalist, 1883(17): p. 319-320.

18. Burse, A., H. Weingart, and M.S. Ullrich. NorM, an *Erwinia amylovora* multidrug efflux pump involved in in vitro competition with other epiphytic bacteria. Applied and Environmental Microbiology, 2004. **70**(2): p. 693-703.

19. Chatterjee, S., N. Killiny, R.P.P. Almeida, and S.E. Lindow. Role of cyclic di-GMP in *Xylella fastidiosa* biofilm formation, plant virulence, and insect transmission. Molecular Plant-Microbe Interactions, 2010. **23**(10): p. 1356-1363.

20. Christen, B., M. Christen, R. Paul, F. Schmid, M. Folcher, P. Jenoe, M. Meuwly, and U. Jenal. Allosteric control of cyclic di-GMP signaling. The Journal of Biological Chemistry, 2006. **281**(42): p. 32015-24.

21. Costerton, J.W. Bacterial biofilms: A common cause of persistent infections. Science, 1999. **284**(5418): p. 1318-1322.

22. Cotter, P. and S. Stibitz. C-di-GMP-mediated regulation of virulence and biofilm formation. Current Opinion in Microbiology, 2007. **10**(1): p. 17-23.

23. D'Argenio, D.A., M.W. Calfee, P.B. Rainey, and E.C. Pesci. Autolysis and autoaggregation in *Pseudomonas aeruginosa* colony morphology mutants. Journal of Bacteriology, 2002. **184**(23): p. 6481-6489.

24. Datsenko, K.A. and B.L. Wanner. One-step inactivation of chromosomal genes in *Escherichia coli* k-12 using PCR products. Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(12): p. 6640-5.

25. DebRoy, S., R. Thilmony, Y.B. Kwack, K. Nomura, and S.Y. He. A family of conserved bacterial effectors inhibits salicylic acid-mediated basal immunity and promotes disease necrosis in plants. Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(26): p. 9927-9932.

26. Delorenzo, V. and K.N. Timmis. Analysis and construction of stable phenotypes in gram-negative bacteria with tn5-derived and tn10-derived minitransposons, in Bacterial Pathogenesis, Pt A1994. p. 386-405.

27. Donlan, R.M. and J.W. Costerton. Biofilms: Survival mechanisms of clinically relevant microorganisms. Clinical Microbiology Reviews, 2002. **15**(2): p. 167-193.

28. Dunn, A.K., D.S. Millikan, D.M. Adin, J.L. Bose, and E.V. Stabb. New *rfp*- and pES213-derived tools for analyzing symbiotic *Vibrio fischeri* reveal patterns of infection and *lux* expression In Situ. Applied and Environmental Microbiology, 2006. **72**(1): p. 802-10.

29. Eden-Green, S.J. and E. Billing. Fireblight. Review of Plant Pathology, 1974(53): p. 353-365.

30. Ehrlich, G.D., F.Z. Hu, and J.C. Post, eds. Role for biofilms in infectious disease. Microbial Biofilms, ed. M. Ghannoum and G.A. O'Toole2004, ASM Press: Washington D.C.

31. Ferreira, R.B.R., L.C.M. Antunes, E.P. Greenberg, and L.L. McCarter. *Vibrio parahaemolyticus* ScrC modulates cyclic dimeric GMP regulation of gene expression relevant to growth on surfaces. Journal of Bacteriology, 2007. **190**(3): p. 851-860.

32. Finn, R.D., J. Mistry, J. Tate, P. Coggill, A. Heger, J.E. Pollington, O.L. Gavin, P. Gunasekaran, G. Ceric, K. Forslund, L. Holm, E.L. Sonnhammer, S.R. Eddy, and A. Bateman. The pfam protein families database. Nucleic Acids Research, 2010. **38**(Database issue): p. D211-22.

33. Galan, J.E. Molecular genetic bases of *Salmonella* entry into host cells. Molecular Microbiology, 1996. **20**(2): p. 263-271.

34. Galperin, M.Y. Bacterial signal transduction network in a genomic perspective. Environmental Microbiology, 2004. **6**(6): p. 552-67.

35. Gaudriault, S., L. Malandrin, J.P. Paulin, and M.A. Barny. DspA, an essential pathogenicity factor of *Erwinia amylovora* showing homology with AvrE of

Pseudomonas syringae, is secreted via the Hrp secretion pathway in a DspB-dependent way. Molecular Microbiology, 1997. **26**(5): p. 1057-1069.

36. Geider, K. Twenty years of molecular genetics with *Erwinia amylovora*: Answers and new questions about Eps-synthesis and other virulence factors. Acta Horticulture, 2006(704): p. 397-402.

37. Glawe, D.A. Thomas j. Burrill, pioneer in plant pathology. Annual Review of Phytopathology, 1992(30): p. 17-24.

38. Hall-Stoodley, L., J.W. Costerton, and P. Stoodley. Bacterial biofilms: From the natural environment to infectious diseases. Nature Reviews, Microbiology, 2004. **2**(2): p. 95-108.

39. Hall-Stoodley, L., L. Nistico, K. Sambanthamoorthy, B. Dice, D. Nguyen, W.J. Mershon, C. Johnson, F.Z. Hu, P. Stoodley, G.D. Ehrlich, and J.C. Post. Characterization of biofilm matrix, degradation by DNase treatment and evidence of capsule downregulation in *Streptococcus pneumoniae* clinical isolates. BMC Microbiology, 2008. **8**: p. 173.

40. Hayward, A.C., ed. *Pseudomonas solanacearum*. Pathogenesis and host-parasite specificity in plant diseases: Histopathological, biochemical, genetic and molecular bases, ed. U.S. Singh, R.P. Singh, and K. Khomoto. Vol. 1. 1995, Pergamon Press: Oxford. 21-46.

41. Hengge, R. Principles of c-di-GMP signalling in bacteria. Nature Reviews, Microbiology, 2009. **7**(4): p. 263-73.

42. Hickman, J.W. A chemosensory system that regulates biofilm formation through modulation of cyclic diguanylate levels. Proceedings of the National Academy of Sciences, 2005. **102**(40): p. 14422-14427.

43. Huang, P.Y. and R.N. Goodman. Ultrastructural modifications in apple stems induced by *Erwinia amylovora* and the fire blight toxin. Phytopathology, 1976(66): p. 269-276.

44. Huynh, T.V., D. Dahlbeck, and B.J. Staskawicz. Bacterial-blight of soybean - regulation of a pathogen gene determining host cultivar specificity. Science, 1989. **245**(4924): p. 1374-1377.

45. Jenal, U. and J. Malone. Mechanisms of cyclic-di-GMP signaling in bacteria. Annual Review of Genetics, 2006. **40**: p. 385-407.

46. Jumel, K., K. Geider, and S.E. Harding. The solution molecular weight and shape of the bacterial exopolysaccharides amylovoran and stewartan. International Journal of Biological Macromolecules, 1997. **20**(4): p. 251-258.

47. Kelm, O., C. Kiecker, K. Geider, and F. Bernhard. Interaction of the regulator proteins RcsA and RcsB with the promoter of the operon for amylovoran biosynthesis in *Erwinia amylovora.* Molecular & General Genetics, 1997. **256**(1): p. 72-83.

48. Koczan, J.M., M.J. McGrath, Y. Zhao, and G.W. Sundin. Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: Implications in pathogenicity. Phytopathology, 2009. **99**(11): p. 1237-44.

49. Koutsoudis, M.D. Quorum-sensing regulation governs bacterial adhesion, biofilm development, and host colonization in *Pantoea stewartii* subspecies *stewartii.* Proceedings of the National Academy of Sciences, 2006. **103**(15): p. 5983-5988.

50. Krogh, A., B. Larsson, G. von Heijne, and E.L.L. Sonnhammer. Predicting transmembrane protein topology with a hidden markov model: Application to complete genomes. Journal of molecular biology, 2001. **305**(3): p. 567-580.

51. Kulesekara, H. Analysis of *Pseudomonas aeruginosa* diguanylate cyclases and phosphodiesterases reveals a role for bis-(3'-5')-cyclic-GMP in virulence. Proceedings of the National Academy of Sciences, 2006. **103**(8): p. 2839-2844.

52. Lenz, D.H., K.C. Mok, B.N. Lilley, R.V. Kulkarni, N.S. Wingreen, and B.L. Bassler. The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae.* Cell, 2004. **118**(1): p. 69-82.

53. Long, T., K.C. Tu, Y.F. Wang, P. Mehta, N.P. Ong, B.L. Bassler, and N.S. Wingreen. Quantifying the integration of quorum-sensing signals with single-cell resolution. PLoS Biology, 2009. **7**(3): p. 640-649.

54. Longstroth, M. The firebliht epidemic in southwest michigan 2000, Michigan State University Extension.

55. Macnab, R.M. The bacterial flagellum: Reversible rotary propellor and type III export apparatus. Journal of Bacteriology, 1999. **18**(23): p. 7149-7153.

56. Meng, X.D., J.M. Bonasera, J.F. Kim, R.M. Nissinen, and S.V. Beer. Apple proteins that interact with DspA/E, a pathogenicity effector of *Erwinia amylovora*, the fire blight pathogen. Molecular Plant-Microbe Interactions, 2006. **19**(1): p. 53-61.

57. Merritt, J.H., K.M. Brothers, S.L. Kuchma, and G.A. O'Toole. SadC reciprocally influences biofilm formation and swarming motility via modulation of exopolysaccharide production and flagellar function. Journal of Bacteriology, 2007. **189**(22): p. 8154-64.

58. Momol, M.T., J.L. Norelli, D.E. Piccioni, E.A. Momol, H.L. Gustafson, J.N. Cummins, and H.S. Aldwinckle. Internal movement of *Erwinia amylovora* through symptomless apple scion tissues into the rootstock. Plant Disease, 1998(82): p. 646-650.

59. Nimtz, M., A. Mort, T. Domke, V. Wray, Y.X. Zhang, F. Qiu, D. Coplin, and K. Geider. Structure of amylovoran, the capsular exopolysaccharide from the fire blight pathogen *Erwinia amylovora.* Carbohydrate Research, 1996. **287**: p. 59-76.

60. Norelli, J.L., A.L. Jones, and H.S. Aldwinckle. Fire blight management in the twenty-first century: Using new technologies that enhance host resistance in apple. Plant Disease, 2003. **87**(7): p. 756-765.

61. Parsek, M.R. and P.K. Singh. Bacterial biofilms: An emerging link to disease pathogenesis. Annual Review of Microbiology, 2003. **57**: p. 677-701.

62. Paul, R., S. Weiser, N.C. Amiot, C. Chan, T. Schirmer, B. Giese, and U. Jenal. Cell cycle-dependent dynamic localization of a bacterial response regulator with a novel di-guanylate cyclase output domain. Genes & Development, 2004. **18**(6): p. 715-727.

63. Payne, S.M. and R.A. Finkelstein. Detection and differentiation of iron-responsive avirulent mutants on congo red agar. Infection and Immunity, 1977. **18**(1): p. 94-98.

64. Politis, D.J. and R.N. Goodman. Fine-structure of extracellular polysaccharide of *Erwinia amylovora.* Applied and Environmental Microbiology, 1980. **40**(3): p. 596-607.

65. Ramey, B.E., M. Koutsoudis, S.B.v. Bodman, and C. Fuqua. Biofilm formation in plant–microbe associations. Current Opinion in Microbiology, 2004. **7**(6): p. 602-609.

66. Rao, F., Y. Yang, Y. Qi, and Z.X. Liang. Catalytic mechanism of cyclic di-GMP-specific phosphodiesterase: A study of the EAL domain-containing RocR from *Pseudomonas aeruginosa.* Journal of Bacteriology, 2008. **190**(10): p. 3622-31.

67. Raymundo, A.K. and S.M. Ries. Chemotaxis of *Erwinia amylovora.* Physiology and Biochemistry, 1980(70): p. 1066-1069.

68. Raymundo, A.K. and S.M. Ries. Motility of *Erwinia amylovora.* Ecology and Epidemiology, 1981(75): p. 45-49.

69. Rigano, L.A., F. Siciliano, R. Enrique, L. Sendin, P. Filippone, P.S. Torres, J. Questa, J.M. Dow, A.P. Castagnaro, A.A. Vojnov, and M.R. Marano. Biofilm formation, epiphytic fitness, and canker development in *Xanthomonas axonopodis* pv *citri.* Molecular Plant-Microbe Interactions, 2007. **20**(10): p. 1222-1230.

70. Romling, U. Molecular biology of cellulose production in bacteria. Research in Microbiology, 2002. **153**(4): p. 205-212.

71. Romling, U. and D. Amikam. Cyclic di-GMP as a second messenger. Current Opinion in Microbiology, 2006. **9**(2): p. 218-28.

72. Romling, U., M. Gomelsky, and M.Y. Galperin. C-di-GMP: The dawning of a novel bacterial signalling system. Molecular Microbiology, 2005. **57**(3): p. 629-39.

73. Romling, U., M. Rohde, A. Olsen, S. Normark, and J. Reinkoster. AgfD, the checkpoint of multicellular and aggregative behaviour in *Salmonella typhimurium* regulates at least two independent pathways. Molecular Microbiology, 2000. **36**(1): p. 10-23.

74. Ross, P., Y. Aloni, C. Weinhouse, D. Michaeli, P. Weinbergerohana, R. Meyer, and M. Benziman. An unusual guanyl oligonucleotide regulates cellulose synthesis in *Acetobacter-xylinum.* Febs Letters, 1985. **186**(2): p. 191-196.

75. Ross, P., Y. Aloni, H. Weinhouse, D. Michaeli, P. Weinbergerohana, R. Mayer, and M. Benziman. Control of cellulose synthesis in *Acetobacter-xylinum* - a unique guanyl oligonucleotide is the immediate activator of the cellulose synthase. Carbohydrate Research, 1986. **149**(1): p. 101-117.

76. Ross, P., H. Weinhouse, Y. Aloni, D. Michaeli, P. Weinbergerohana, R. Mayer, S. Braun, E. Devroom, G.A. Vandermarel, J.H. Vanboom, and M. Benziman. Regulation of cellulose synthesis in *Acetobacter-xylinum* by cyclic diguanylic acid. Nature, 1987. **325**(6101): p. 279-281.

77. Ryan, R.P., Y. Fouhy, J.F. Lucey, L.C. Crossman, S. Spiro, Y.W. He, L.H. Zhang, S. Heeb, M. Camara, P. Williams, and J.M. Dow. Cell-cell signaling in *Xanthomonas campestris* involves an HD-GYP domain protein that functions in cyclic di-GMP turnover. Proceedings of the National Academy of Sciences of the United States of America, 2006. **103**(17): p. 6712-7.

78. Ryan, R.P., Y. Fouhy, J.F. Lucey, B.L. Jiang, Y.Q. He, J.X. Feng, J.L. Tang, and J.M. Dow. Cyclic di-GMP signalling in the virulence and environmental adaptation of *Xanthomonas campestris.* Molecular Microbiology, 2007. **63**(2): p. 429-42.

79. Ryan, R.P., Y. McCarthy, M. Andrade, C.S. Farah, J.P. Armitage, and J.M. Dow. Cell-cell signal-dependent dynamic interactions between HD-GYP and GGDEF domain proteins mediate virulence in *Xanthomonas campestris.* Proceedings of the National Academy of Sciences of the United States of America, 2010. **107**(13): p. 5989-94.

80. Ryjenkov, D.A., M. Tarutina, O.V. Moskvin, and M. Gomelsky. Cyclic diguanylate is a ubiquitous signaling molecule in bacteria: Insights into biochemistry of the GGDEF protein domain. Journal of Bacteriology. 2005. **187**(5): p. 1792-8.

81. Sambrook, J., E.F. Fritsch, and T. Maniatis, Molecular cloning: A laboratory manual. 1989, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

82. Sasakura, Y., S. Hirata, S. Sugiyama, S. Suzuki, S. Taguchi, M. Watanabe, T. Matsui, I. Sagami, and T. Shimizu. Characterization of a direct oxygen sensor heme protein from *Escherichia coli*. Effects of the heme redox states and mutations at the heme-binding site on catalysis and structure. The Journal of Biological Chemistry, 2002. **277**(26): p. 23821-7.

83. Schmidt, A.J., D.A. Ryjenkov, and M. Gomelsky. The ubiquitous protein domain EAL is a cyclic diguanylate-specific phosphodiesterase: Enzymatically active and inactive EAL domains. Journal of Bacteriology, 2005. **187**(14): p. 4774-81.

84. Scott, J.E. Methods of Biochemical Analysis, 1960(8): p. 145-197.

85. Sebaihia, M., A.M. Bocsanczy, B.S. Biehl, M.A. Quail, N.T. Perna, J.D. Glasner, G.A. DeClerck, S. Cartinhour, D.J. Schneider, S.D. Bentley, J. Parkhill, and S.V. Beer. Complete genome sequence of the plant pathogen *Erwinia amylovora* strain ATCC 49946. Journal of Bacteriology, 2010. **192**(7): p. 2020-2021.

86. Semuller, E.A. and S.V. Beer. Absence of cell wall polysaccharide degradation by *erwinia amylovora.* Physiology and Biochemistry, 1976(66): p. 433-436.

87. Simm, R., J.D. Fetherston, A. Kader, U. Romling, and R.D. Perry. Phenotypic convergence mediated by GGDEF-domain-containing proteins. Journal of Bacteriology, 2005. **187**(19): p. 6816-23.

88. Simm, R., M. Morr, A. Kader, M. Nimtz, and U. Romling. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. Molecular Microbiology, 2004. **53**(4): p. 1123-34.

89. Sjulin, T.M. and S.V. Beer. Mechanism of wilt induction by amylovorin in cotoneaster shoots and its relation to wilting of shoots infected by *Erwinia amylovora.* Phytopathology, 1978. **68**(1): p. 89-94.

90. Solano, C., B. Garcia, C. Latasa, A. Toledo-Arana, V. Zorraquino, J. Valle, J. Casals, E. Pedroso, and I. Lasa. Genetic reductionist approach for dissecting individual roles of GGDEF proteins within the c-di-GMP signaling network in *Salmonella.* Proceedings of the National Academy of Sciences of the United States of America, 2009. **106**(19): p. 7997-8002.

91. Steinberger, E.M. and S.V. Beer. Creation and complementation of pathogenicity mutants of *Erwinia amylovora.* Molecular Plant-Microbe Interactions, 1988. **1**(3): p. 135-144.

92. Sutherland, I.W. Biofilm exopolysaccharides: A strong and sticky framework. Microbiology-UK, 2001. **147**: p. 3-9.

93. Tagliabue, L., D. Antoniani, A. Maciag, P. Bocci, N. Raffaelli, and P. Landini. The diguanylate cyclase YddV controls production of the exopolysaccharide poly-N-acetylglucosamine (PNAG) through regulation of the PNAG biosynthetic *pgaABCD* operon. Microbiology, 2010. **156**(Pt 10): p. 2901-11.

94. Tal, R., H.C. Wong, R. Calhoon, D. Gelfand, A.L. Fear, G. Volman, R. Mayer, P. Ross, D. Amikam, H. Weinhouse, A. Cohen, S. Sapir, P. Ohana, and M. Benziman. Three *cdg* operons control cellular turnover of cyclic di-GMP in *Acetobacter xylinum*: Genetic organization and occurrence of conserved domains in isoenzymes. Journal of Bacteriology, 1998. **180**(17): p. 4416-4425.

95. Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 2011.

96. Tischler, A.D. and A. Camilli. Cyclic diguanylate (c-di-GMP) regulates *Vibrio cholerae* biofilm formation. Molecular Microbiology, 2004. **53**(3): p. 857-69.

97. Tischler, A.D. and A. Camilli. Cyclic diguanylate regulates *Vibrio cholerae* virulence gene expression. Infection and Immunity, 2005. **73**(9): p. 5873-82.

98. Triplett, L.R., M. Melotto, and G.W. Sundin. Functional analysis of the N terminus of the *Erwinia amylovora* secreted effector DspA/E reveals features required for secretion, translocation, and binding to the chaperone DspB/F. Molecular Plant-Microbe Interactions, 2009. **22**(10): p. 1282-1292.

99. Vanneste, J.L., ed. *Erwinia amylovora*. In Pathogenesis and host specificity in plant diseases: Histopathological, biochemical, genetic and molecular bases. Vol. 1. 1995, Pergammon Press: Oxford and London.

100. Vanneste, J.L., ed. Fire blight: The disease and its causative agent, *Erwinia amylovora*. 2000, CABI Publishing: New Zealand.

101. Waters, C.M. and B.L. Bassler. The Vibrio harveyi quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. Genes & Development, 2006. **20**(19): p. 2754-67.

102. Waters, C.M., W. Lu, J.D. Rabinowitz, and B.L. Bassler. Quorum sensing controls biofilm formation in *Vibrio cholerae* through modulation of cyclic di-GMP levels and repression of VpsT. Journal of Bacteriology, 2008. **190**(7): p. 2527-36.

103. Wehland, M., C. Kiecker, D.L. Coplin, O. Kelm, W. Saenger, and F. Bernhard. Identification of an RcsA/RcsB recognition motif in the promoters of exopolysaccharide biosynthetic operons from *Erwinia amylovora* and *Pantoea stewartii* subspecies *stewartii.* Journal of Biological Chemistry, 1999. **274**(6): p. 3300-3307.

104. Wilson, M., H.A.S. Epton, and D.C. Sigee. *Erwinia amylovora* infection of hawthorn blossom: II. The stigma. Journal of Phytopathology, 1989(127): p. 15-28.

105. Wilson, M., D.C. Sigee, and H.A.S. Epton. *Erwinia amylovora* infection of hawthorn blossom: III. The nectary. Journal of Phytopathology, 1990(128): p. 62-74.

106. Wolfe, A.J. and K.L. Visick. Get the message out: Cyclic-di-GMP regulates multiple levels of flagellum-based motility. Journal of Bacteriology, 2008. **190**(2): p. 463-75.

107. Wolfe, A.J. and K.L. Visick, eds. The second messenger cyclic di-GMP. Moshe Benziman and the discovery of cyclic di-GMP, ed. D. Amikam, H. Weinhouse, and M.Y. Galperin2010, ASM Press: Washington, D.C.

108. Yi, X., A. Yamazaki, E. Biddle, Q. Zeng, and C.-H. Yang. Genetic analysis of two phosphodiesterases reveals cyclic diguanylate regulation of virulence factors in *Dickeya dadantii.* Molecular Microbiology, 2010. **77**(3): p. 787-800.

109. Zhao, Y., S.E. Blumer, and G.W. Sundin. Identification of *Erwinia amylovora* genes induced during infection of immature pear tissue. Journal of Bacteriology, 2005. **187**(23): p. 8088-103.

110. Zhao, Y., G.W. Sundin, and D. Wang. Construction and analysis of pathogenicity island deletion mutants of *Erwinia amylovora.* Canadian Journal of Microbiology, 2009. **55**(4): p. 457-64.

111. Zobell, C.E. The effect of solid surfaces upon bacterial activity. Journal of Bacteriology, 1943. **46**(1): p. 39-56.