CENTRAL CARBON METABOLISM REGULATES CELLULOSE PRODUCTION IN THE PLANT PATHOGEN *ERWINIA AMYLOVORA*

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ABSTRACT

The bacterium *Erwinia amylovora* is a gram-negative pathogen of rose family plants worldwide and is the causal agent of the devastating fire blight disease, causing substantial damage to apple and pear trees annually. Cellulose is an exopolysaccharide produced by *Erwinia amylovora* from host carbohydrates that improves the three-dimensional structure of biofilms in the xylem of apple host plants, contributing to the devastating fire blight disease. Cellulose biosynthesis is regulated by the second messenger c-di-GMP, which is produced and degraded by diguanylate cyclases (DGCs) and specific phosphodiesterases (PDEs), respectively. While several DGCs and PDEs have the potential to contribute to cellulose production, the specificity of these enzymes is poorly known. In this study, host carbohydrates were added to growth media *in vitro*, and knockout mutant strains were used to examine the specificity of PDEs and a DGC with respect to cellulose production under carbohydrate-rich conditions. Experimental results indicate that glucose, sucrose, and sorbitol each resulted in an increase in cellulose production, which caused bacteria to engage in autoaggregation behavior in liquid medium. The *pdeC* gene had the strongest negative impact on cellulose production out of the *E. amylovora* PDEs, while mutation of *dgcB* abolished cellulose production. A mutant strain unable to metabolize sorbitol was unable to produce cellulose on sorbitol-containing medium, and sugar fermentation occurred in all sugar-enriched media, indicating that fermentative metabolism may favor cellulose production in specific host environments such as the xylem.

This work is dedicated to Naomi
and also Martin, Julie, Ronald, and Pauline

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LIST OF SYMBOLS

α - alpha

β - beta

μ - micro

Δ - delta

LIST OF ABBREVIATIONS

c-di-GMP – cyclic dimeric guanosine monophosphate

CFU – colony forming unit

DGC – diguanylate cyclase

EPS – exopolysaccharide

LB – Luria-Bertani medium or Lysogeny Broth

PDE - phosphodiesterase

ROS – reactive oxygen species

sRNA – small RNA

T3SS – type 3 secretion system

TCA – tricarboxylic acid

TCS – two component system

Chapter 1. Introduction and Literature Review

1.1. Fire blight disease and the causal agent *Erwinia amylovora*

The Gram-negative bacterium *Erwinia amylovora* has been named one of the top 10 most important plant pathogenic bacteria for causing fire blight disease on apple, pear, and other Rosaceous plants (Mansfield et al., 2012). One of the first bacterial pathogens known, *E. amylovora* is a member of the family *Enterobacteriaceae*, similar to other plant and animal pathogens (Piqué et al., 2015). The disease caused by this bacterium is complex and devastating, with severe outbreaks leading to the death of entire apple and pear trees and costing local and global economies millions of dollars annually (Norelli et al., 2003). The disease cycle begins in spring, when *E. amylovora* is transferred to the flower stigma by wind, rain, or insects. Populations on the stigma can grow up to 1 x 10^6 CFU/flower before migrating to the floral cup and entering the plant through the nectaries (Slack et al., 2022). Systemic infection then occurs, with bacterial population within the plant causing 'scorched' symptoms on flowers, leaves, and shoots. *E. amylovora* forms biofilms in the xylem tissue of leaves and shoots, blocking water flow and contributing to the distinctive symptoms (Koczan et al., 2009). Secondary infections can occur via wounds and natural openings. Eventually, the bacteria form cankers on the trunk and crown of the tree, which can cause tree death and also provides an overwintering site for the pathogen (Norelli et al., 2003). Periodically throughout the disease cycle, masses of bacterial cells, exopolysaccharides (EPS), and sugars will burst through the plant tissue to form ooze droplets on the surface, which serve as a source of inoculum for further infection (Bennett & Billing, 1980; Slack et al., 2017).

1.2. Virulence factors of *E. amylovora*

During the various infection stages on different host tissues, *E. amylovora* deploys a number of virulence factors to cause disease. During flower colonization, flagellar motility is

important for the migration of the bacteria from the stigma into the hypanthium of the flower (Schachterle, Zeng, et al., 2019). The bacteria also induce a ROS burst from the host immune system, but are able to overcome the oxidative effects due to the activity of catalase enzymes (Schachterle, Onsay, et al., 2019). In addition to catalases, the siderophore desferrioxamine has shown to be a key factor contributing to survival of ROS production in *E. amylovora*, and also plays a key role in scavenging bioavailable iron, a limiting nutrient in apple flowers (Dellagi et al., 1998).

An important virulence factor in *E. amylovora* is the ability to metabolize host carbohydrates. In the apple host, several carbohydrates are consumed over the course of the disease cycle. Analysis of the stigma tip revealed that glucose and fructose contributed to sigma colonization, while in the nectar, sucrose is the primary sugar (Pusey et al., 2008). Interestingly, while sucrose is the primary transport carbohydrate in most plants, apple, pear, and other hosts of *E. amylovora* contain sorbitol as the main carbohydrate (Aldridge et al., 1997). While glucose and fructose can be immediately processed via glycolysis, sucrose and sorbitol require specific enzymes to transport and convert these sugars into glycolytic intermediates. The five-gene *scr* operon is responsible for sucrose uptake and metabolism in *E. amylovora*, and deletion of these genes reduced disease symptoms in apple seedlings (Bogs & Geider, 2000).Similarly, the *srl* operon encodes six genes that phosphorylate and translocate sorbitol molecules into the cell, where sorbitol-6-phosphate 2-dehydrogenase (SrlD) converts sorbitol-6-phosphate to fructose-6-phosphate, which is then able to be further processed by glycolysis (Salomone-Stagni et al., 2018). Mutant strains lacking the *srl* operon were found to have greatly decreased virulence in apple plants, highlighting the importance of sorbitol metabolism in fire blight disease development (Aldridge et al., 1997).

A facultative anaerobe, *E. amylovora* can metabolize carbon using oxidative metabolism and the Tricarboxilic Acid (TCA) cycle, or fermentative metabolism. Fermentation pathways may be very important for *E. amylovora* growth and survival, especially in anaerobic or semi-anaerobic conditions. A study conducted on *E. amylovora* glucose metabolism in aerobic and semi-anaerobic conditions found that, in both conditions, ethanol, lactate, and carbon dioxide were the primary metabolic products (Sutton & Starr, 1959). In addition, smaller amounts of acetic, formic, and succinic acid were also detected in this study. The high yields of ethanol formation reported by this study and others, even in aerobic conditions, can be explained by the presence of pyruvate decarboxylase and ethanol dehydrogenase enzymes in *E. amylovora*. In fact, the thiamine pyrophosphate-dependent pyruvate decarboxylase is quite rare among bacteria, and *E. amylovora* was the first member of the *Enterobacteriaceae* reported to possess this enzyme (Haq & Dawes, 1971). The fermentative pathway for carbon substrates seems to be an important factor for growth *in vitro* for this pathogen, and researchers have speculated that the bacteria may encounter different oxygen levels as they move from flower and shoot surfaces to more interior tissues such as xylem (Schachterle, Onsay, et al., 2019). How carbon metabolism affects the regulation of other virulence factors in *E. amylovora* remains poorly understood.

One of the most important virulence factors in *E. amylovora* is the type III secretion system (T3SS), used to translocate effector proteins into the host. These effectors suppress host immunity and cause cell death, and electrolyte leakage, contributing to the cells' survival of reactive oxygen species (ROS) produced by the host during early infection (Piqué et al., 2015). A single effector, DspA/E, is largely responsible for these properties (Boureau et al., 2006). Equally important for pathogenicity is the EPS amylovoran, a heteropolymer of glucose, galactose, and pyruvate (Koczan et al., 2009). Mutagenesis of the *ams* gene cluster, responsible

for the biosynthesis of amylovoran, renders the bacteria unable to infect the host (Bellemann & Geider, 1992). Amylovoran is an important component of biofilms, contributing to host colonization, and is the major constituent of bacterial ooze, an important stage in the disease cycle (Koczan et al., 2009; Slack et al., 2017). *E. amylovora* also produces the EPS levan, a homopolymer of fructose, which also contributes to biofilm formation and is found in ooze and together with amylovoran contributes to sliding motility (Koczan et al., 2009; Slack et al., 2017; Yuan, Eldred, & Sundin, 2022). A third EPS, cellulose, has been more recently discovered to play a role in virulence. Cellulose has been shown to be an important component of xylem biofilms, contributing to their three-dimensional structure, and is important for full virulence *in planta* (Castiblanco & Sundin, 2018). Interestingly, cellulose was not found to contribute to sliding motility in *E. amylovora*, in contrast to amylovoran and levan (Yuan, Eldred, & Sundin, 2022). It is currently unknown if cellulose is only produced in xylem biofilms or is involved with other aspects of the fire blight disease cycle.

1.3. Bacterial cellulose production

Cellulose is one of the most abundant biological polymers on Earth and is the primary component of the cell wall in plants (Abidi et al., 2022). By no means limited to plants, however, cellulose is produced by a wide diversity of bacteria as well (Römling & Galperin, 2015). Although cellulose in its purest form is a homopolymer of glucose molecules connected by β-1,4 linkages, there are differences in the properties of bacterial and plant cellulose. For example, cellulose from bacterial sources has been noted to display much higher purity, crystallinity, malleability, and porosity than plant cellulose, which can contain similar polymers with different bond structures such as lignin and hemicellulose (Naomi et al., 2020). These characteristics, in addition to high tensile strength, biodegradability, and low toxicity, have led to great interest in

various applications of bacterial cellulose, including wound healing, drug delivery, tissue grafting, toxin removal, soil additives, and even as a thickener and stabilizing agent in foods (Mohite & Patil, 2014).

Bacterial cellulose polymers, as well as other bacterial exopolysaccharides, are key constituents of biofilms, and can play a fundamental role in the biology of many pathogenic bacteria. Cellulose is a component of *Salmonella* and *Escherichia coli* biofilms, contributing to cell-cell interaction, macrocolony structure, biofilm architecture, and resistance to environmental stresses (Römling & Galperin, 2015; Serra et al., 2013). Due to its insolubility and high hydrophilicity, cellulose has significant capacity to trap and retain water. In *Es. coli*, cellulose production increased resistance to osmotic stress and chlorine treatment in pathogenic strain O157, and another study found exopolysaccharide overproduction in response to osmotic stress in strain K-12 (Ionescu & Belkin, 2009; Yoo & Chen, 2010). Plant-pathogenic bacteria *Dickya dadantii* and *Agrobacterium tumefaciens* produce cellulose to aid colonization of plant surfaces, and cellulose production enhances phyllosphere persistence in epiphytic *Pseudomonas fluorescens* (Römling & Galperin, 2015). In *E. amylovora*, cellulose was discovered to play a major role in the architecture of xylem biofilms, modulating the three-dimensional structure *in vitro* and *in planta* (Castiblanco & Sundin, 2018). This study also reported that strains unable to produce cellulose were less virulent on apple shoots, demonstrating the importance of cellulose as a virulence factor in a shoot blight infection model.

Bacterial cellulose synthase (Bcs) proteins are responsible for the synthesis and translocation of bacterial cellulose. These Bcs proteins form a multiprotein complex spanning the inner and outer membranes and are encoded by the *bcs* operon, typically consisting of three to seven genes (Römling & Galperin, 2015). The protein products of *bcsA* and *bcsB* are essential

for cellulose production, while the other associated *bcs* genes are involved in regulation, transport and translocation of the cellulose polymer, or have uncharacterized functions (Römling & Galperin, 2015). According to proposed classification, *E. amylovora* contains a Type 1b *bcs* operon, containing the periplasmic protein *bcsD* as well as the putative endogluconase *bcsZ* (Castiblanco & Sundin, 2018; Römling & Galperin, 2015).

The Bcs system was famously discovered to be allosterically activated by the ubiquitous second messenger molecule Cyclic dimeric Guanosine Monophosphate (c-di-GMP). The BcsA protein is one of the most well-studied subunits of the Bcs system because it contains a C-terminal cytoplasmic PilZ domain, a known receptor for c-di-GMP (Ryjenkov et al., 2006). Upon allosteric binding of c-di-GMP to the PilZ domain of BcsA, an intermolecular salt bridge is broken which releases an inhibitory gating loop, resulting in access of the UDP-glucose substrate to the enzymatic active site of BcsA (Morgan et al., 2014). Because of the nature of the BcsA gating loop, the Bcs complex is constitutively inactive in the absence of c-di-GMP. In *E. amylovora*, c-di-GMP strongly regulates cellulose production by binding to the PilZ domain on BcsA. Mutation of a key amino acid in this binding site resulted in a lack of cellulose production, even in the presence of high c-di-GMP levels (Castiblanco & Sundin, 2018).

1.4. Cyclic di GMP signaling in *E. amylovora*

Cyclic dimeric Guanosine Monophosphate (c-di-GMP) is a ubiquitous second messenger molecule found in a diverse array of bacteria. First discovered in the 1970's as an activator of cellulose biosynthesis in *Acetobacter xylinum*, c-di-GMP has since become known as one of the most complex and important prokaryotic signal transduction mechanisms, and genes related to c-di-GMP metabolism have been found in all major bacterial phyla (Römling et al., 2013; Ross et al., 1973). Many important cellular functions, including motility, cell division, differentiation,

virulence, and biofilm formation are regulated by this key molecule (Römling et al., 2013). Composed of two GMP molecules, c-di-GMP forms a relatively stable 12-member ribose-phosphate ring structure, and is synthesized by diguanylate cyclase enzymes (DGCs). While often having a diverse domain architecture, DGCs all share a GGDEF domain, named for the conserved enzymatically active site responsible for the synthesis of c-di-GMP (Ausmees et al., 2001). The GGDEF domain is enzymatically active as a homodimer, and the other components of DGC proteins often have a significant effect on the ability of the proteins to dimerize, influencing the amount of c-di-GMP output in the cell. Conversely, c-di-GMP is degraded by specific phosphodiesterase enzymes (PDEs), which contain either an EAL or HD-GYP domain that contains the active site. The more common EAL domain linearizes the c-di-GMP molecule to the degradation product 5'-pGpG, whereas the HD-GYP domain is able to complete hydrolysis and produce two molecules of GMP (Bobrov et al., 2005; Galperin et al., 1999). The EAL domain does not require dimerization for activity, unlike the GGDEF domain, but its activity is often increased as a dimer (Bobrov et al., 2005). Interestingly, tandem enzymes containing both a GGDEF and an EAL domain appear to be relatively common. Some of these are truly bifunctional with the ability to both synthesize and degrade c-di-GMP, but more often one of the two domains is inactive due to a degenerate active site motif (Römling et al., 2013; Tarutina et al., 2006).

While the GGDEF, EAL, and HD-GYP domains are conserved across different bacterial lineages, the overall protein architecture of DGCs and PDEs is highly variable. Many bacterial species have up to several dozen various c-di-GMP enzymes, indicating that the variety of different protein structures plays a key role in c-di-GMP signal transduction. For example, the model organism *Escherichia coli* contains 29 DGC and PDE genes with a variety of sensory

domains and proteins structures (Hengge et al., 2016). DGCs and PDEs can be part of one-component, two-component, or chemosensory systems, and may be cytoplasmic or membrane-bound, often with N-terminal sensory domains (Randall et al., 2022). The sensory domains percieve a variety of external stimuli such as temperature, light, nutrients, oxidative state, molecular oxygen levels, quorum sensing molecules, and others (Almblad et al., 2021; Enomoto et al., 2023; Herbst et al., 2018; Huang et al., 2013; Rossi et al., 2022; Waldron et al., 2019). Transmitting these signals into a c-di-GMP output, c-di-GMP enzymes may affect the entire cell globally, or may provide more localized signal transduction that only affects a specific downstream process (Reinders et al., 2016; Richter et al., 2020). However, even with these recent advances in knowledge, most of the specific signals affecting the expression or function of individual DGCs or PDEs remain a mystery, and many of the sensory domains are poorly characterized.

Five DGC enzymes (*edcA, edcB, edcC, edcD, edcE*) have been characterized in the genome of *E. amylovora*, possessing active GGDEF domains with all residues necessary for c-di-GMP synthesis (Edmunds et al., 2013). Except for *edcA*, each of the enzymes is predicted to be membrane-associated with multiple transmembrane domains, and *edcA, edcD,* and *edcE* each have predicted sensory domains at the N-terminus. In contrast, *edcC* and *edcB* do not contain a predicted sensory domain, and only have multiple transmembrane domains and the GGDEF domain. Overexpression of *edcB. edcC, edcD,* and *edcE* each increased intracellular c-di-GMP levels, amylovoran production, and biofilm formation while decreasing swimming motility (Edmunds et al., 2013). In addition, *edcB, edcC,* and *edcD* were shown to upregulate cellulose production in *E. amylovora* (Castiblanco & Sundin, 2018). This study also suggests that, while c-di-GMP produced from DgcC and DgcE can activate cellulose biosynthesis in *E. amylovora*,

DgcB may be more specifically related to cellulose, as overexpression of this enzyme increased the production of cellulose but not amylovoran. In addition, a transposon mutagenesis experiment implicated DgcB as a key positive regulator of cellulose production in *E. amylovora*, but the factors regulating the expression of the *dgcB* gene as well as the regulation of the PDE enzymes remain unknown (Yuan, Eldred, Kharadi, et al., 2022).

E. amylovora also contains three active PDE enzymes (*pdeA*, *pdeB*, and *pdeC*). All three PDEs have active EAL domains and are predicted to be membrane-associated, with *pdeA* and *pdeB* containing an N-terminal CSS sensory domain commonly found in PDE enzymes (Kharadi et al., 2019a). Interestingly, *pdeC* has a more complex protein architecture with a predicted N-terminal GAPES3 sensory domain, a HAMP domain usually involved in transmitting conformational changes, and a degenerate GGDEF domain lacking the required active site residues for c-di-GMP synthesis. The importance or function of the GAPES3 domain is not known, even though it is found in other important pathogens such as *Yersinia pestis*. Mutagenesis studies by Kharadi et al., (2019) revealed that PdeC is the most active PDE in *E. amylovora*, with a knockout mutant showing significantly higher intercellular c-di-GMP levels. However, all three PDE knockouts displayed similar increases in biofilm formation and amylovoran production, while not appearing to significantly affect swimming motility. A triple mutant lacking all three PDE genes showed the highest c-di-GMP levels, significantly increasing amylovoran production while decreasing motility, ooze production, *hrp* gene expression, and virulence on immature pears. This triple mutant also autoaggregated in liquid culture, a phenotype associated with sessile growth and biofilm formation. The lack of distinct phenotype in the *pdeC* single mutant, even with significantly increased c-di-GMP levels, is interesting and could indicate that *pdeC* plays a more specific role in other aspects of virulence. However, the

effects of *pdeC* on other virulence factors, such as cellulose production, have not yet been investigated.

Further delving into the complexity of c-di-GMP signaling in *E. amylovora*, (Kharadi et al., 2022a) constructed a strain missing all five active DGCs and all three active PDEs (Δ8) and another strain missing an additional four c-di-GMP related genes (Δ12). The authors discovered that, despite high expression of the T3SS, the Δ12 strain is unable to effectively colonize the apple shoot and is deficient in surface attachment and biofilm formation. In addition, the transcriptome was substantially altered in the absence of c-di-GMP signaling genes, with over 500 genes differentially expressed, including genes for metabolic pathways and other regulatory systems. Each DGC contributed to biofilm formation when complemented into the Δ8 strain, suggesting that effective host colonization requires the concerted orchestration of all DGCs in *E. amylovora*.

Biofilm formation has been one of the most well-studied c-di-GMP regulated processes, and biofilms have also been implicated as an important part of the fire blight disease cycle (Kharadi & Sundin, 2021). A related behavior in *E. amylvora* is autoaggregation, in which visible bacterial clumps settle to the bottom of liquid culture media. Research on autoaggregation of this pathogen discovered that the autoaggregation process is highly c-di-GMP dependent, but does not necessarily correlate with biofilm formation. In fact, mutant strains that had high levels of c-di-GMP and displayed autoaggregation showed greatly decreased biofilm formation (Kharadi & Sundin, 2019). The authors attribute this interesting result to the increased propensity for cell-cell interaction compared to cell-surface interaction. In addition, autoaggregation also involved cell division genes *sulA* and *ftsZ*, with decreased expression of the cytoskeletal *ftsZ* gene. One of the key factors contributing to autoaggregation in *E. amylovora* is EPS production,

specifically the EPSs amylovoran and cellulose. Strains unable to produce one or both EPSs showed marked decreases in autoaggregation, despite high c-di-GMP levels (Kharadi & Sundin, 2019). These cell-cell interactions are likely important for forming biofilm structures and establishing and maintaining sessile growth.

1.5. Other mechanisms of virulence regulation

While c-di-GMP is one of the most important mechanisms of virulence gene regulation, bacteria use a variety of other systems to alter virulence strategies and adapt to changing environments. Two-component systems (TCS) are commonly found signal transduction mechanisms usually consisting of a receptor kinase, which perceives a signal and phosphorylates the response regulator, which then performs a specific function. Response regulators are often enzymatically active or are transcriptional regulators, but may have other functions. Analysis of the *E. amylovora* genome revealed forty-six predicted TCS genes, and mutagenesis of many of these had significant effects on virulence factors such as amylovoran production and swarming motility, and impacted virulence on immature pear fruit (Zhao, Wang, et al., 2009). In particular, the EnvZ/OmpR TCS was discovered to positively regulate amylovoran production and swarming motility. EnvZ is well known in other organisms as a sensor of acidic pH and changes in osmotic pressure, and has a pleiotropic effect on the cell. Conversely, the GrrS/GrrA TCS negatively regulated amylovoran and swarming motility in *E. amylovora* (Zhao, Wang, et al., 2009).The Rcs system, a phosphorelay containing a TCS, has been demonstrated to be critical in regulating amylovoran production. Mutations of the *rcs* genes resulted in a lack of virulence in immature pear fruit (D. Wang et al., 2009). Other important TCS in *E. amylovora* include the ArcA/B system, known in other organisms to regulate anaerobic metabolism, the PhoP/Q system, sensitive to environmental magnesium and pH levels, and the HrpX/Y system, involved

in the T3SS (Zhao, Wang, et al., 2009).

TCS and c-di-GMP are important mechanisms by which bacteria perceive and respond to the environment, but in recent years small noncoding RNAs (sRNA) have emerged as critical regulators of virulence in many pathogens. These 50-100 nucleotide molecules of RNA play diverse roles in regulating gene expression and protein activity. Most commonly, sRNAs act as antisense RNA that regulates mRNA transcripts by complementary binding. Many bacterial sRNAs require the chaperone protein Hfq for full stability and activity. In other instances, sRNAs may act as riboswitches or bind to proteins to directly alter their function (Gottesman & Storz, 2011). *E. amylovora* contains up to 40 Hfq-dependent sRNAs, several of which have important functions in virulence (Zeng & Sundin, 2014). For example, the sRNA RmaA negatively regulates amylovoran production and positively regulates swimming motility, and the sRNA ArcZ regulates a variety of processes including biofilm formation, cell-surface attachment, T3SS, amylovoran production, and motility (Schachterle, Zeng, et al., 2019; Zeng et al., 2013). Additionally, ArcZ is important for surviving ROS produced by the host immune system by regulating catalase genes in *E. amylovora*, highlighting the importance of this sRNA in causing disease (Schachterle, Onsay, et al., 2019).

Carbon metabolism could be a key interface in the regulation of various virulence factors. A recent study of *E. amylovora* discovered that thiamine availability and biosynthesis increased production of the pathogenicity factor amylovoran (Yuan et al., 2021). Another interesting result from that study is that enzymes involved with oxidative metabolism greatly decreased amylovoran production. The EPS cellulose is one of the more recently discovered virulence factors in *E. amylovora*, and the regulation of cellulose in response to environmental conditions and potential roles in virulence could provide insight into bacterial signal transduction and plant

disease.

1.6. Objectives

In this study, my objectives are: 1) evaluate the specificity of DGC and PDE enzymes that regulate cellulose production, and 2) the mode of regulation for these enzymes. I also investigate 3) potential metabolic pathways that could facilitate cellulose production, and explore the effects of cellulose on bacterial behavior.

Chapter 2. Fermentative sugar metabolism stimulates cellulose production in *Erwinia amylovora*

2.1. Abstract

Cellulose is an exopolysaccharide produced by *Erwinia amylovora* that improves the three-dimensional structure of biofilms in the xylem of apple host plants, contributing to the devastating fire blight disease. Cellulose is composed of glucose molecules, and is energetically expensive for bacteria to produce. However, the relationship between carbon metabolism and cellulose production in *Erwinia amylovora* remains understudied. Cellulose biosynthesis is regulated by the second messenger c-di-GMP, which is produced and degraded by diguanylate cyclases (DGCs) and specific phosphodiesterases (PDEs), respectively. While several DGCs and PDEs have the potential to contribute to cellulose production, the specificity of these enzymes is poorly known. In this study, host carbohydrates were added to growth media *in vitro*, and knockout mutant strains were used to examine the specificity of PDEs and a DGC with respect to cellulose production under carbohydrate-rich conditions. Experimental results indicate that glucose, sucrose, and sorbitol each resulted in an increase in cellulose production, which caused bacteria to engage in autoaggregation behavior in liquid medium. The *pdeC* gene had the strongest negative impact on cellulose production out of the *E. amylovora* PDEs, while mutation of *dgcB* abolished cellulose production. A mutant strain unable to metabolize sorbitol was unable to produce cellulose on sorbitol-containing medium, and sugar fermentation occurred in all sugar-enriched media. Mutations of enzymes in the Tricarboxylic Acid (TCA) cycle decreased, but did not abolish, cellulose production, indicating that fermentative metabolism may favor cellulose production compared to other EPS such as amylovoran.

2.2. Introduction

The Gram-negative plant pathogen *Erwinia amylovora* causes the destructive fire blight disease on rose family plants, most notably apple and pear. The bacteria are able to navigate through a wide variety of plant parts and tissues during the disease cycle, and they employ a variety of virulence mechanisms to do so. In the spring, bacteria emerge from trunk and stem cankers as ooze which is then transferred to flower stigmas by insects, wind, and rain (Slack et al., 2017). After building up large populations epiphytically, *E. amylovora* uses flagellar motility to migrate to the floral cup and enter the nectarthodes. Once inside the plant, *E. amylovora* can systemically infect shoot and stem tissue, periodically bursting forth with masses of exopolysaccharides (EPS) as ooze droplets which initiate secondary infections on growing shoot tips (Slack et al., 2022). Once inside the xylem of the host plant, *E. amylovora* attaches to the xylem wall and forms biofilms, which can slow water flow and contribute to wilting symptoms and disease development (Koczan et al., 2009).

E. amylovora produces three EPS: amylovoran, which is a pathogenicity factor important for all infection stages; levan, which is a component of ooze droplets and biofilms while contributing to swarming motility; and cellulose, which is important for the three-dimensional structure of biofilms (Yuan, Eldred, & Sundin, 2022; Yuan, Eldred, Kharadi, et al., 2022). Cellulose, synthesized by the Bcs complex, is regulated by the second messenger cyclic-dimeric guanosine monophosphate (c-di-GMP), which allosterically activates BcsA in *E. amylovora* (Koczan et al., 2009). The c-di-GMP molecule is synthesized by diguanylate cyclases (DGCs) and degraded by phosphodiesterases (PDEs) (Kharadi et al., 2019b). In other bacteria, such as *Escherichia coli* and *Yersinia pestis*, a specific DGC/PDE enzyme pair regulates the c-di-GMP levels near the Bcs system, thus controlling cellulose production (Bobrov et al., 2005; Richter et

al., 2020). DGCs and PDEs are well known for their diversity of roles in bacterial signal transduction, and often contain signaling domains that perceive external cues and modulate c-di-GMP levels accordingly (Randall et al., 2022). Of particular importance in the change from a motile, planktonic lifestyle to a sessile, biofilm lifestyle, c-di-GMP is a critical mechanism that *E. amylovora* uses to adapt virulence strategies to the specific host environment (Kharadi et al., 2022b).

Mutations that alter c-di-GMP levels can have drastic effects on bacterial behavior *in vitro*. For example, mutagenesis of the three PDEs in *E. amylovora* resulted in high c-di-GMP levels, which increased biofilm formation and caused autoaggregation behavior, when cells and EPS clump together in culture media (Kharadi & Sundin, 2019). Conversely, strains that lacked one of the DGCs showed greatly decreased biofilm formation (Edmunds et al., 2013). Previous studies of DGCs in *E. amylovora* discovered that overexpression of several individual DGC enzymes each increased cellulose production, and a separate transposon mutagenesis study revealed there may be a specific effect of DgcB on the regulation of cellulose in *E. amylovora* (Koczan et al., 2009; Yuan, Eldred, Kharadi, et al., 2022). The specificity of PDE enzymes on cellulose production in *E. amylovora* has not yet been explored. However, a mutation of *pdeC* increased intracellular c-di-GMP levels without having a distinct effect on amylovoran production and biofilm formation compared to mutation of other active PDE enzymes in *E. amylovora* (Kharadi et al., 2019b). This could potentially indicate that PdeC regulates some other process, such as cellulose production.

EPSs such as cellulose are a key factor in autoaggregation and biofilm formation, and offer stability and protection for *E. amylovora* during sessile stages of infection. Composed of glucose polymers, production of cellulose necessitates that host carbohydrates be directed from

bacterial metabolism to EPS synthesis, representing a significant energy cost to the pathogen. However, how availability of host carbohydrates influences cellulose production has not been studied.

An important virulence factor in *E. amylovora* is the ability to metabolize host carbohydrates. Analysis of the stigma tip revealed that glucose and fructose contributed to sigma colonization while in the nectar sucrose is the primary sugar. Sorbitol, however is the most abundant carbohydrate in apple plants, especially in shoot and leaf tissues (Pusey et al., 2008).Mutant strains lacking the ability to metabolize sorbitol were found to have greatly decreased virulence in apple plants, highlighting the importance of sorbitol metabolism in fire blight disease development (Aldridge et al., 1997). While these various carbohydrates are abundant in the apple host tissues, the common LB growth medium used for *in vitro* studies of *E. amylovora* does not have a significant amount of carbohydrates (Kharadi et al., 2019b). In this study, we investigate the effect of carbohydrate metabolism on cellulose production *in vitro*, and observe the impact of cellulose production on bacterial behavior. In addition, we hypothesize that DgcB is an essential positive regulator of cellulose production, and that PdeC negatively regulates cellulose production. We also investigate mutagenesis of the signaling domain of PdeC, and propose potential regulatory pathways for these enzymes.

2.3. Materials and Methods

The bacterial strains and plasmids used in this study are compiled in Table 1. Unless otherwise noted, *E. amylovora* and *Escherichia coli* were grown in LB broth at 28° or 37° C, respectively. Appropriate antibiotics were added to growth media at the following concentrations: ampicillin (Ap) at 100 μg/mL, chloramphenicol (Cm) at 10 μg/mL, gentamicin (Gm) at 15 μg/mL, and kanamycin (Km) at 30 μg/mL. The genome sequence of *E. amylovora* ATCC 49946 was obtained from GenBank (accession no. FN666575) and used to determine sequences of oligonucleotide primers used for the construction of mutants and complementation clones (Sebaihia et al., 2010).

Table 1. Table of all bacterial strains and plasmids used in this study. Strain names in the left column are followed by relevant characteristics and reference publications.

Strain name	**Characteristics**	**Source**
Ea1189	Wild type	(Yuan, Eldred, Kharadi, et al., 2022)
Δ*bcsA*	*bcsA* deletion mutant Ea1189	(Castiblanco & Sundin, 2018)
Δ*pdeA*	*pdeA* deletion mutant Ea1189	(Kharadi et al., 2019b)
Δ*pdeB*	*pdeB* deletion mutant Ea1189	(Kharadi et al., 2019b)
Δ*pdeC*	*pdeC* deletion mutant Ea1189	(Kharadi et al., 2019b)
Δ8	*pdeA, pdeB, pdeC, dgcA, dgcB, dgcC, dgcD, dgcE* combination deletion mutant Ea1189	(Kharadi et al., 2022b)
Δ*dgcB*	*dgcB* deletion mutant Ea1189	(Yuan, Eldred, Kharadi, et al., 2022)

Table 1. (cont'd)

Δsrl	*srlAEBDMR* operon mutant Ea1189	This study
Δ*aceE*	*aceE* deletion mutant Ea1189	(Yuan et al., 2021)
Δ*sucA*	*sucA* deletion mutant Ea1189	(Yuan et al., 2021)
Escherichia coli DH5α	Cloning strain	Lab Stock
Plasmid name	**Characteristics**	**Source**
pBBR1-MCS5:: *pdeC*	*pdeC* cloned into pBBR1-MCS5; Gm^r	(Kharadi et al., 2019b)
pLFC34::*dgcB*	*dgcB* overexpression vector with IPTG-inducible promoter; Km^r	(Castiblanco & Sundin, 2018)
pKD46	Arabinose-inducible lambda red recombinase, Ap^r	(Datsenko & Wanner, 2000)
pKD3	Cm^R cassette flanked by FRT sites	(Datsenko & Wanner, 2000)
pBAD	pBAD LIC 8A plasmid, Ap^r	AddGene commercial vector

Bioinformatics

Accessions EAM_RS16620 and EAM_RS02755 were used to find sequences for *pdeC* and *dgcB* in the National Center for Biotechnology Information Gene database (NCBI Gene), respectively, and homology to genes from other organisms was assessed by aligning genes using the NCBI BLAST tool (Edmunds et al., 2013; Kharadi et al., 2019). Protein domain structure was analyzed using SMART database, and amino acid sequence alignments were generated using the MEGA version 7.0 program (Finn et al., 2016; Tamura et al., 2013).

Genetic manipulations

Chromosomal mutants were constructed in *E. amylovora* using the lambda red recombinase method previously described (Datsenko & Wanner, 2000; Yuan, Eldred, Kharadi, et al., 2022; Zhao, Sundin, et al., 2009). In summary, primers were designed with a target gene-specific overhang attached to flanking sequences of the short flippase recognition target (FRT)

sites of the Cm resistance cassette from the plasmid pKD3. The Cm resistance cassette was amplified with these primers from the pKD3 plasmid, which after gel electrophoresis were purified using a QIAEX II Gel Extraction Kit (Qiagen, Hilden, Germany). Purified PCR products were electroporated into *E. amylovora* cells containing the helper plasmid pKD46 and plated onto LB medium supplemented with Cm to select for chromosomal insertions of the Cm cassette. Validation that the correct mutation occurred was confirmed with PCR using primers outside the mutagenized region and confirmed with Sanger sequencing. For complementation of target genes, the open reading frame (ORF) of the target gene was amplified via PCR and cloned into the complementation or overexpression vector. The correct insertion was confirmed with Sanger sequencing and the plasmid was electroporated into the appropriate *E. amylovora* strain.

A truncated *pdeC* gene was designed by designing primers to start after the N-terminal GAPES3 domain region of the *pdeC* gene and insert a start codon, creating a shortened *pdeC* gene with a start codon that was cloned into an arabinose-inducible plasmid vector.

Domain-directed mutagenesis was performed on the GAPES3 domain of *pdeC* using the EZ Clone GeneMorph II Domain Mutagenesis Kit (Agilent) according to manufacturer instructions. In brief, error-prone polymerases were used to amplify the GAPES3 region of the *pdeC* gene, and the product of this PCR was used as a megaprimer to resynthesize the pBBR1-MCS5::*pdeC* plasmid, with the result of an intact plasmid and full-length *pdeC* gene with a randomly mutagenized GAPES3 domain. Results were analyzed via Sanger sequencing.

Cellulose production assays

Cellulose production of *E. amylovora* strains was observed using saltless LB medium amended with 40 µg/mL Congo Red dye and 20 µg/mL Coomassie Brilliant Blue dye, as previously described (Castiblanco & Sundin, 2018; Yuan, Eldred, Kharadi, et al., 2022). *E.*

amylovora cells were grown overnight in LB medium to an optical density at 600 nm ($O.D_{600}$) of 0.5 and spotted onto Congo Red medium in 5 µL aliquots. Plates were incubated at 28° for 24 hours before images were taken using a Leica M165 C microscope equipped with a DFC295 camera. Cellulose production was quantified from these images as per previous studies (Yuan, Eldred, Kharadi, et al., 2022). To measure colony redness, representing cellulose production, images were split into 3 RGB (red, green, and blue) channels using ImageJ and the resulting green images were chosen to quantify the redness. Grayscale densities of the entire bacterial colonies were quantified using the default settings of ImageJ. In order to qualitatively assess the presence of cellulose in liquid cell culture, cell cultures were washed with sterile water and stained with a mixture of Calcofluor white and Evans blue at concentrations of 1 g/L and 0.5 g/L, respectively. After overnight staining, cells were washed three times with sterile water, placed in a clear 24-well plate, and photographed under UV light to visually observe the fluorescence of Calcofluor bound to cellulose (Castiblanco & Sundin, 2018). Washed cell cultures and aggregates were also placed in a 0.1 M potassium phosphate buffer (pH 5) and exposed to 10 u/mL purified fungal cellulase enzyme (*Trichoderma viride* cellulase, Sigma-Aldrich, Germany) and shaken gently at 37° C to assess sensitivity of cell aggregates to cellulase, similar to previous studies (Jahn et al., 2011; Yap et al., 2005). Aggregates with and without exposure to cellulase were photographed at 0 and 120 minutes to observe any degradation. Experiments were repeated three times, with three technical replicates per experiment.

Determination of aggregation factor

Methods from previous studies of autoaggregation in *E. amylovora* were used to calculate the aggregation factor (Kharadi & Sundin, 2019). In brief, bacterial strains were grown for 20 h at 28°. A 200 µL sample of growing medium was removed from the culture. Following this, the

cultures were vortexed for 30 s, and 200 µL of growing medium was removed from the culture. The aggregation factor was calculated by determining the OD_{600} ratio of the medium removed post-homogenization to that removed pre-homogenization. Experiments were repeated three times, with three technical replicates per experiment.

Gene expression analysis

E. amylovora RNA was extracted using a Zymo RNA Clean and Concentrator kit (Research Products International, USA) according to the manufacturer's instructions. Genomic DNA was removed using Turbo DNA-free DNase (Ambion, Austin, TX, USA), and cDNA was synthesized using SuperScript III First-Strand synthesis system (Invitrogen, CA, USA). To evaluate the mRNA levels of *bcsA*, *dgcB*, and *pdeC*, quantitative real-time PCR (qRT-PCR) was conducted using cDNA as the template. Levels of cDNAs from different samples were quantified using SYBR green PCR master mix (Applied Biosystems, Foster City, CA, USA), and the relative expression levels of each target gene were calculated using the $2^{-\Delta\Delta CT}$ method, with the *recA* gene as the internal control (Livak & Schmittgen, 2001; Takle et al., 2007). Primers used for qRT-PCR are listed in Table 2. Experiments were repeated three times.

Table 2. Primer names, sequences, and sources of all primers used in this study.

Primer	**Sequence**	**Source**
srl mutagenesis F	5' –ATGATTGAAGCTATCA CACATGGGGCCGAATG GTTTATCGGTCTTTTCCAGTGTAGGCTGGAGCTGCTTC – 3'	This study
srl mutagenesis R	5' –TCAGTCCTCTCCTGCAGTGATGACGCTGATAT TCATCCCTGACAGCTGTTCATATGAATATCCTCCTTA - 3'	This study
pdeC qPCR F	5'- TTACGGTAATGGTGCTGCGT- 3'	This study
pdeC qPCR R	5'- GCTGCGAATTTTGCTCACCA - 3'	This study
dgcB qPCR F	5'- GCGCTTATCTTCTGGTTGCG - 3'	This study

Table 2. (cont'd)

dgcB qPCR R	5'- GATCGAAGTCGGTGCCGTAT - 3'	This study
bcsA qPCR F	5'- AACCACGCCATGCAAATCAC - 3'	(Yuan, Eldred, Kharadi, et al., 2022)
bcsA qPCR R	5'- AGTAGTGCGGCGTCTGTAAC - 3'	(Yuan, Eldred, Kharadi, et al., 2022)
pdeC cloning primer with overhang F	5'- ttaactttaagaaggagatatagatTTGCGCGTCAGCCGTTCATTAAAGA - 3'	This study
pdeC cloning primer R	5'- caagggatccttatggagttgggatTCAGTAACTGGCCAGATAGCGCT - 3'	This study
pBAD cloning site primer F	5'- atgccatagcatttttatcc- 3'	This study
pBAD cloning site primer R	5'- GATTTAATCTGTATCAGG - 3'	This study
Truncated *pdeC* cloning primer F	5'- ttaactttaagaaggagatatagatTTGGCTGTATCGACGATGGTCACC - 3'	This study
Truncated *pdeC* cloning primer F	5'- ttaactttaagaaggagatatagatTTGGCTGTATCGACGATGGTCACC - 3'	This study
Truncated *pdeC* cloning primer R	5'- caagggatccttatggagttgggatTCAGTAACTGGCCAGATAGCGCT - 3'	This study
pBBr1-MCS M13 F	5'- CCCAGTCACGACGTTGTAAAACG - 3'	This study
pBBr1-MCS M13 R	5'- AGCGGATAACAATTTCACACAGG - 3'	This study
GAPES3 domain region F	5'- TTTATCGTTATCCAGCTTTTTCA - 3'	This study

Table 2. (cont'd)

GAPES3 domain region R	5'- GATAGGCGGTGACCATCG - 3'	This study
pBAD cloning site primer F	5'- atgccatagcatttttatcc- 3'	This study
pBAD cloning site primer R	5'- GATTTAATCTGTATCAGG - 3'	This study
GAPES3 domain region F	5'- TTTATCGTTATCCAGCTTTTTCA - 3'	This study
GAPES3 domain region R	5'- GATAGGCGGTGACCATCG - 3'	This study

Analysis of sorbitol fermentation ability using MacConkey medium

MacConkey indicator plates (10 g/L peptone, 2.5 g/L NaCl, 30 mg/L Phenol Red) with 1% sorbitol were used to qualitatively analyze the ability of the mutant strains to ferment sorbitol. Phenol Red in the medium serves as a pH indicator; sorbitol fermentation is signaled by color change from red (> ~7.5) to yellow (< ~6.8) with intermediate shades of orange.

C-di-GMP quantification

Intracellular levels of c-di-GMP were quantified using ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS), as described previously (Massie et al., 2012). Overnight cultures were diluted 1:30 and grown until they reached an OD_{600} of 0.8 to 1.0 and then normalized to an OD_{600} of 0.7, 5 ml of this new culture was pelleted, and the supernatant was discarded. The pelleted cells were treated with 100 µL of an extraction buffer solution (40% acetonitrile and 40% methanol) and incubated overnight at 20° C, after which 10 µL of supernatant was analyzed by UPLC-MS/MS on a Waters TQ-S instrument. All samples were quantified by comparison against a standard curve generated by using synthetic c-di-GMP (Axxora

Life Sciences Inc., San Diego, CA). This assay was repeated three times.

2.4. Results

Addition of host sugars stimulates cellulose production and autoaggregation in E. amylovora*.*

Glucose, sucrose, and sorbitol are the primary free carbohydrates found in the apple and pear host plants of *E. amylovora*, and are often found at concentrations up to 80 mg/mL (Wang & Stutte, 1992). When 20 mg/mL of these three host carbohydrates were added to LB agar medium, we observed that all three host sugars resulted in significantly brighter red microcolonies when assayed for cellulose production, indicating that sugars stimulated cellulose production in WT *E. amylovora*. . In contrast, the Δ*bcsA* mutant strain appeared unaffected by the addition of sugars to the growth medium and displayed a lack of cellulose production (Fig.1A). When WT cells were grown in liquid LB medium amended with 20 mg/mL sugars, the cells remained clumped in aggregates on the bottom of the culture tube or clinging to the side of the culture tube, whereas in unamended LB medium *E. amylovora* grew planktonically, creating a turbid culture. To quantify this, the autoaggregation factor was measured, and amendment of LB medium with each of the three sugars resulted in a significant increase in the autoaggregation factor of the WT strain. Similarly to the cellulose assay, the Δ*bcsA* mutant strain appeared unaffected by the addition of sugars to the growth medium, showing planktonic growth in the presence or absence of sugars (Fig.1B). This suggests that cellulose is a key component of the sugar-stimulated autoaggregation behavior in *E. amylovora*. In addition, the washed cell aggregates fluoresced bluish white when stained with Calcofluor white, and the aggregates were sensitive to enzymatic digestion with cellulase, further supporting this point (Fig.1C-D). The aggregates from all three sugar treatments completely degraded within 120 minutes after exposure to cellulase, suggesting that cellulose was the primary structural component.
All three sugars tested produced very similar phenotypic results in our experiments. Since

sorbitol has been previously demonstrated to be the most abundant transport carbohydrate in apple tissue, and the ability of *E. amylovora* to metabolize sorbitol is an important virulence factor contributing to fire blight disease (Aldridge et al., 1997; Z. Wang & Stutte, 1992), we chose to focus on the effects of sorbitol on signal transduction in *E. amylovora* for the remainder of this study.

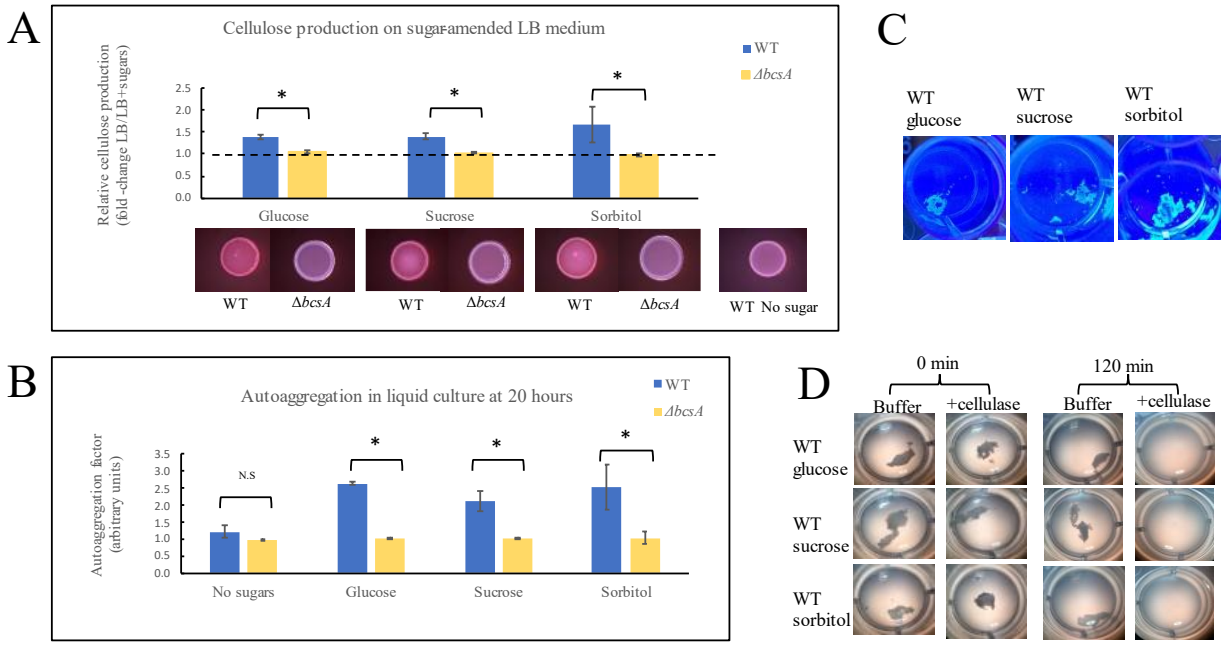

Figure 1. Addition of host sugars stimulates cellulose production and autoaggregation in *E. amylovora*. A) Cellulose production by Ea1189 WT and *bcsA* mutant strains on Congo Red LB agar medium amended with 20 mg/mL sugars. Quantification of macrocolony redness was measured with WT on unamended medium as a reference and denoted by the dotted line. B) Quantification of autoaggregation factor in liquid LB medium amended with sugars after 20 hours of growth. Error bars signify standard deviation; presence of an asterisk above the bars indicates statistically significant difference observed ($P > 0.05$ by Student's *t* test). C) Calcoflour white binding on DI-washed cellulose aggregates produced by Ea1189 WT, visualized under UV light. Background fluorescence is due to Evans Blue dye added to the reagent for contrast. D) Enzymatic digestion of DI-washed cellulose aggregates by cellulase over time.

PdeC and DgcB are the main cyclic-di-GMP enzymes involved in cellulose production in E. amylovora.

E. amylovora contains 8 active cyclic-di-GMP metabolizing enzymes, and we compared these proteins to known regulators of cellulose production in other organisms to predict potential roles of c-di-GMP enzymes in regulating cellulose production. In the widespread human pathogen *Escherichia coli*, the *bcs* operon is associated with a specific diguanylate cyclase and a specific phosphodiesterase that regulate the local pool of cyclic-di-GMP available to activate cellulose synthase (Richter et al., 2020). To predict if *E. amylovora* contains any homologs to these proteins, a protein BLAST search was performed using the amino acid sequences of *Es. coli* proteins DgcC and PdeK (accessions NP_414919.1 and NP_417986.4, respectively) against the genome of *E. amylovora*. These cellulose-associated proteins showed significant homology to the *E. amylovora* proteins DgcB and PdeC. *Es. coli* DgcC (371 amino acids) showed 36.57% sequence similarity to *E. amylovora* DgcB (365 amino acids). *Es. coli* PdeK (649 amino acids) and *E. amylovora* PdeC (664 amino acids) showed 50.45% amino acid sequence similarity with 99% query cover (Appendix 1). In addition, *E. amylovora* DgcB and PdeC also have similar domain structures to corresponding *Es. coli* proteins DgcC and PdeK, with 5 predicted transmembrane helices followed by the GGDEF domain for the DGCs, and a periplasmic domain flanked by two transmebrane helices followed by GGDEF and EAL domains for the PDEs (Appendix 1). These *E. amylovora* proteins are also similar to the HmsT and HmsP proteins in the animal pathogen *Yersinia pestis*, both of which are associated with exopolysaccharide production (Bobrov et al., 2005). The location of the *pdeC* gene in the *E. amylovora* chromosome was also located immediately next to the *bcs* operon, suggesting a role in cellulose production (Appendix 1). Based on this bioinformatic prediction, we hypothesized that DgcB

and PdeC play an important role in cellulose production in *E. amylovora*.

Knockout mutants of each of the three PDE enzymes in *E. amylovora* were assayed for cellulose production. The Δ*pdeA* and Δ*pdeB* deletion mutant strains did not show a visibly different phenotype from either the WT strain or Δ*bcsA*, with whitish macrocolonies observed on Congo Red plates. In contrast, Δ*pdeC* formed a red macrocolony, suggesting that PdeC negatively regulates cellulose production (Fig2.A). Complementation of the *pdeC* gene in the Δ*pdeC* mutant background abolished the cellulose production phenotype, suggesting that PdeC is the most active in regulating cellulose production out of the three *E. amylovora* PDEs. Previous studies of cellulose production in *E. amylovora* have suggested that, out of the five active diguanylate cyclases, DgcB could be most important for the upregulation of cellulose (Castiblanco & Sundin, 2018; Yuan, Eldred, Kharadi, et al., 2022) further confirm this, the *dgcB* gene in a plasmid vector was introduced into a strain containing knockout mutations of all five diguanylate cyclases and all three phosphodiesterases. This Δ8 strain containing only the *dgcB* gene was then assayed for cellulose production. Interestingly, the Δ8 p*dgcB* strain displayed a red macrocolony, providing evidence that out of all *E. amylovora* c-di-GMP enzymes, only DgcB is necessary to stimulate cellulose production (Fig.2A).

Similar results were observed when autoaggregation was measured, with Δ*bcsA,* Δ*pdeA,* and Δ*pdeB* growing planktonically and not forming an aggregate at the bottom of the culture tube. Although a small aggregate was visible in the Δ*pdeC* mutant, this strain had sufficient planktonic growth so as to render the difference in autoaggregation factor statistically insignificant (Fig.2B). The Δ8 p*dgcB* strain had an autoaggregation factor significantly higher than the wild type, suggesting that this DGC alone can result in the transition from planktonic to sessile growth. This strain, as well as the Δ*pdeC* mutant to a much lesser extent, formed a large

visible aggregate (Fig.2C). Washed aggregates fluoresced bluish white after staining with Calcofluor white and were sensitive to cellulase digestion, confirming that the aggregates formed by the mutant strains contained mostly cellulose, similar to the sugar amended medium (Fig.2D,E). No aggregate was formed or able to be collected for the Calcofluor and cellulase assays in the Δ*bcsA*, Δ*pdeA*, Δ*pdeB*, and Δ*pdeC* p*pdeC* strains.

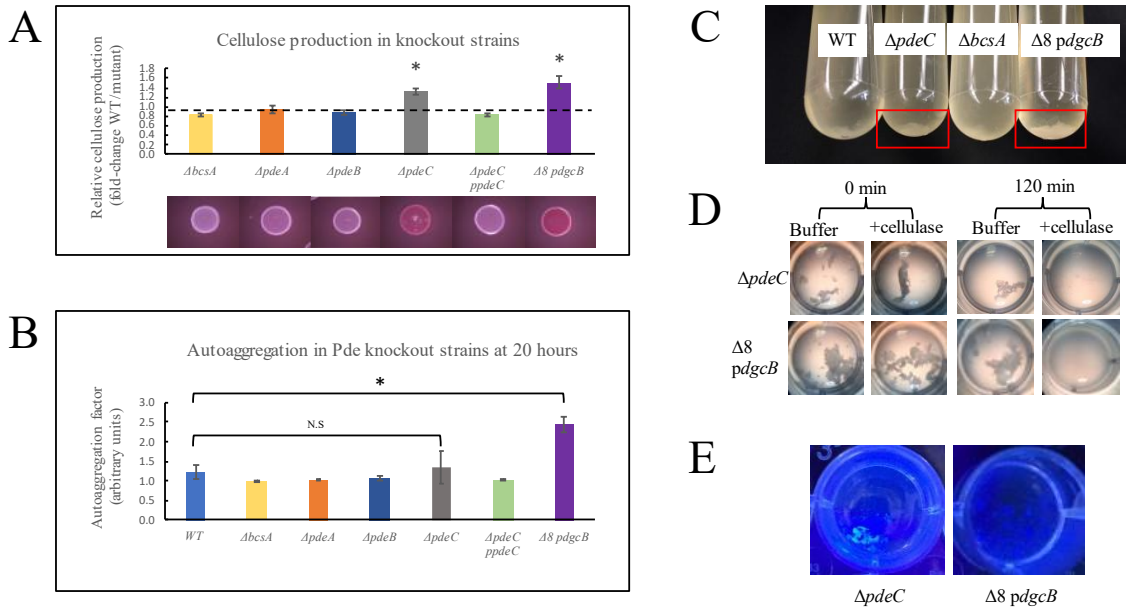

Figure 2. PdeC and DgcB are the main cyclic-di-GMP enzymes involved in cellulose production in *E. amylovora*. A) Cellulose production by Ea1189 mutant strains on Congo Red LB agar medium amended with 20 mg/mL sugars. Quantification of macrocolony redness was measured with WT on unamended medium as a reference and denoted by the dotted line. B) Quantification of autoaggregation factor in liquid LB medium amended with sugars after 20 hours of growth. Error bars signify standard deviation; presence of an asterisk above the bars indicates statistically significant difference observed ($P > 0.05$ by Student's *t* test). C) Image showing visible aggregates settling to the bottom of culture tubes. Red boxes highlight these aggregates. D) Enzymatic digestion of DI-washed cellulose aggregates by cellulase over time. E) Calcoflour white binding on DI-washed cellulose aggregates produced by Ea1189 WT, visualized under UV light. Background fluorescence is due to Evans Blue dye added to the reagent for contrast.

The dgcB *gene is necessary for* E. amylovora *to produce cellulose in response to sugars.*

Since the addition of host sugars resulted in an increase of cellulose production and cellulose-mediated autoaggregation (Fig.1), and the *pdeC* and *dgcB* genes appeared to significantly influence cellulose production and autoaggregation (Fig.2), we hypothesized that these genes were responsible for the phenotype observed upon sugar addition to the growth medium. Mutant strains were assayed for cellulose production and autoaggregation and compared between growth conditions with and without sorbitol. As observed earlier, the WT strain showed significantly higher levels of cellulose production on sorbitol-amended medium, whereas the Δ*bcsA* strain did not appear to produce cellulose in either condition. The Δ*pdeC* strain displayed higher levels of cellulose production in both conditions, both comparable to the WT on sorbitol-amended medium, but there was no significant difference in cellulose production in this strain between conditions (Fig.3A). When this gene was complemented on a plasmid vector, however, there appeared to be a lack of cellulose production even in the presence of sorbitol, and there was no significant difference in cellulose production between conditions for the Δ*pdeC* p*pdeC* strain. These results indicate that the *pdeC* gene is a powerful negative regulator of cellulose production, but may not be specifically involved in the activation of this process in response to host carbohydrates. A single knockout mutation of the *dgcB* gene resulted in a lack of cellulose production, even in the presence of sorbitol, indicating that this enzyme is a critical positive regulator of cellulose production. As before, the Δ8 p*dgcB* strain, expression of this gene alone resulted in high cellulose production, similar to the WT on sorbitol-amended medium and the Δ*pdeC* strain, but there was no significant difference in cellulose production between growth conditions (Fig.3A).

Autoaggregation assays of the mutant strains with and without sorbitol showed a similar

overall trend, with the WT displaying significantly higher autoaggregation with sorbitol and the Δ*bcsA* strain growing planktonically under both conditions. The Δ*pdeC* strain mirrored the phenotype of the WT strain, showing a significantly higher autoaggregation factor when sorbitol was added to the growth medium (Fig.3B). Complementation of this gene abolished the phenotype, with cultures growing planktonically in both conditions. Interestingly, the Δ*dgcB* mutation also abolished autoaggregation (Fig.3B). Together with the cellulose assays, these results suggest that, while PdeC is a key regulator of cellulose biosynthesis in *E. amylovora*, DgcB may be more important in stimulating cellulose production in response to host carbohydrates such as sorbitol.

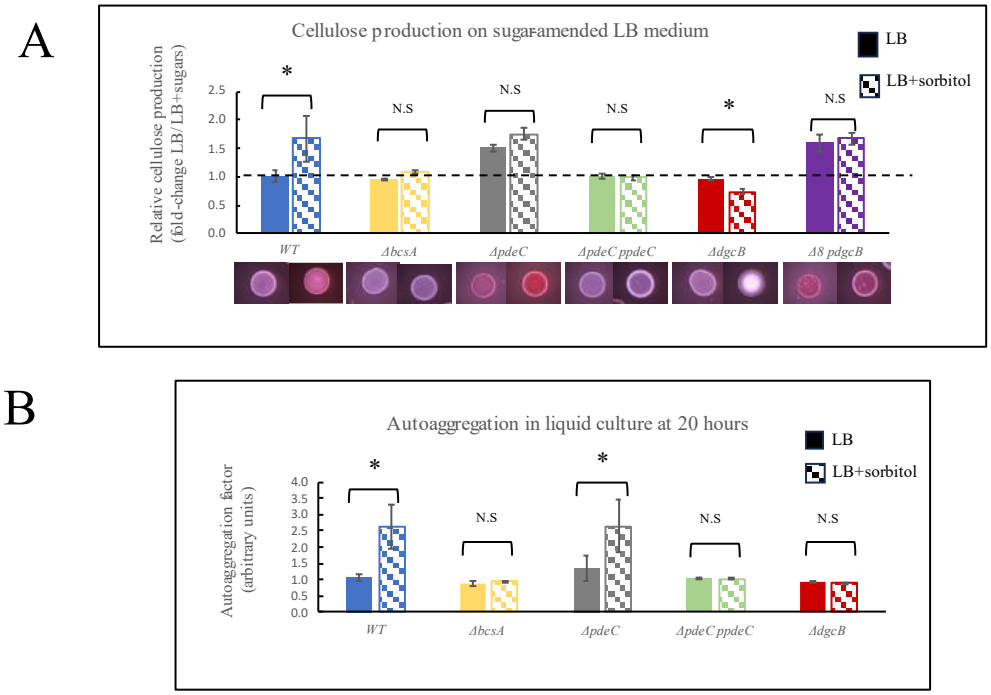

Figure 3. The dgcB gene is necessary for E. amylovora to produce cellulose in response to sugars. A) Cellulose production by Ea1189 mutant strains on Congo Red LB agar medium amended with 20 mg/mL sugars. Quantification of macrocolony redness was measured with WT on unamended medium as a reference and denoted by the dotted line. B) Quantification of autoaggregation factor in liquid LB medium amended with sugars after 20 hours of growth. Error bars signify standard deviation; presence of an asterisk above the bars indicates statistically significant difference observed ($P > 0.05$ by Student's *t* test). Non-significant differences are denoted by N.S.

Addition of sorbitol to LB medium increases expression of dgcB *and intracellular c-di-GMP levels.*

Previous bioinformatic analysis has predicted that DgcB has a relatively simple protein domain architecture, with a C-terminal GGDEF domain and a series of N-terminal transmembrane domains and lacking a predicted sensory domain (Edmunds et al., 2013). This information led us to hypothesize that a key factor in cellulose regulation and associated phenotypes in response to host sugars was the expression of the *dgcB* gene, rather than a protein-level sensory mechanism of the DgcB protein product. We tested the effect of *dgcB* expression induction on autoaggregation using an IPTG-inducible promoter in the Δ8 p*dgcB* strain, so that no other active DGCs or PDEs would be present. When cultures of the Δ8 p*dgcB* strain were grown overnight with no IPTG, the unamended medium showed a higher autoaggregation factor than the sorbitol amended medium. These cultures were then vortexed and IPTG was added to induce *dgcB* expression. Interestingly, within two hours of gene induction the sorbitol-amended culture had formed a dense aggregate, showing a very high autoaggregation factor, whereas the culture without sorbitol had only partially restored autoaggregation behavior upon *dgcB* expression (Fig.4A). These results indicate that expression of dgcB is a primary regulator in the transition from planktonic to sessile growth and biosynthesis of cellulose.

Expression of three target genes, *bcsA, dgcB,* and *pdeC* was measured in the WT strain using qPCR to quantify the effects of sorbitol addition on gene expression. The *bcsA* and *dgcB* genes showed approximately 2.5 and 3-fold increases in expression, respectively (Fig.4B). These results appear to be consistent with the other experiments in this study, as sorbitol increased cellulose production, which requires c-di-GMP as an activator. Unexpectedly, however, the *pdeC* gene was also upregulated about 2-fold (Fig.4B). We found this result surprising because PdeC is

a negative regulator of cellulose production. However, our bioinformatic analysis indicates that the *pdeC* gene is immediately adjacent to the *bcs* operon in *E. amylovora*, and this could potentially explain the co-expression of *bcsA* and *pdeC* (Appendix 1).

Our results suggest that sorbitol increases expression of c-di-GMP metabolizing genes, and we measured intracellular levels of c-di-GMP in cultures grown with or without sorbitol to assess the effect of sorbitol on overall c-di-GMP levels. The WT strain grown in sorbitol-amended medium showed significantly higher c-di-GMP levels, providing further evidence that the presence of sugars induces c-di-GMP mediated cellulose production and autoaggregation (Fig.4C). The *dgcB* mutant strain, however, showed no change in c-di-GMP levels, revealing that this enzyme is responsible for the sorbitol-induced increase in c-di-GMP levels.

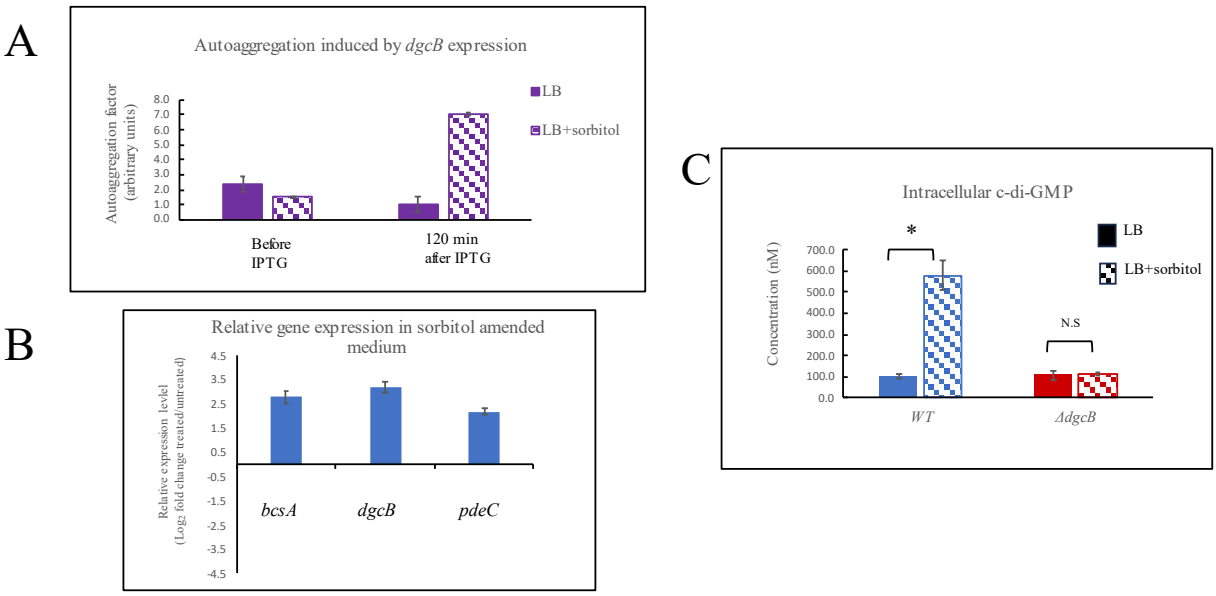

Figure 4. Addition of sorbitol to LB medium increases expression of *dgcB* and intracellular c-di-GMP levels. A) Autoaggregation of Ea1189 Δ8 p*dgcB* mutant strain in liquid LB medium amended with 20 mg/mL sorbitol. The *dgcB* gene was cloned on a plasmid with an IPTG-inducible promoter; autoaggregation measurements were taken before 0.1 mM IPTG was added and two hours after. B) mRNA levels of Ea1189 WT measured by qRT-PCR in liquid LB medium with or without sorbitol after 20 hours of growth. Values are shown relative to endogenous control *recA*. C) Intracellular c-di-GMP concentrations measured with HPLC-MS/MS. Error bars signify standard deviation; presence of an asterisk above the bars indicates statistically significant difference observed ($P > 0.05$ by Student's *t* test). Non-significant differences are denoted by N.S.

Fermentation is associated with cellulose production in E. amylovora.

We observed increases in *dgcB* and *bcsA* expression, c-di-GMP levels, and cellulose-mediated autoaggregation in response to the addition of sorbitol to the growth medium. In light of these results, we questioned whether these phenotypes were the result of sorbitol itself acting as a molecular signal, or via more indirect means through general metabolic changes. To test this, we used a mutant strain lacking the *srl* operon, a six-gene operon necessary for the conversion of sorbitol into glycolytic intermediates, and assayed for sorbitol metabolism and cellulose production. MacConkey indicator plates revealed a pH decrease in response to WT bacterial growth, with the medium changing from magenta to yellow immediately around the bacterial colony after overnight growth. As expected, the *srl* mutant strain was unable to ferment the sorbitol in the medium, and no color change was observed, while the fermentation ability was restored by complementing the *srl* mutant with the whole *srl* operon on a plasmid vector (Fig.5A). The same strains showed the same pattern with respect to cellulose production on sorbitol-amended medium, with the *srl* mutant lacking cellulose production even in the presence of sorbitol, and complementation of this operon restoring cellulose production (Fig.5B). These results suggest that cellulose production requires fermentative sorbitol metabolism under our experimental conditions. Liquid growth medium pH was measured at 18 and 40 hours, and the pH of sorbitol-amended medium decreased by about 1 pH unit, while the pH of unamended medium increased by a similar amount, providing further evidence that sorbitol is being fermented by *E. amylovora* under our experimental conditions (Fig.5C). In addition, while c-di-GMP levels significantly increased with sorbitol addition in the WT strain as observed before, there was not an increase in c-di-GMP in the *srl* mutant strain. These results suggest that the uptake and metabolism of sorbitol are essential for the increase in c-di-GMP that activates

cellulose biosynthesis. Together, these experiments indicate that cellulose production and autoaggregation in response to sorbitol are likely because of sorbitol metabolism, and not some other factor, such as changes in osmotic pressure.

The link between sorbitol fermentation and autoaggregation prompted us to assess the effect of two mutations affecting respiration via the TCA cycle, used in a recent study of *E. amylovora* metabolism (Yuan et al., 2021). These mutations were used to inhibit respiration and completion of the TCA cycle. Interestingly, the Δ*aceE* strain, lacking a pyruvate dehydrogenase gene, did not appear to greatly affect cellulose production or autoaggregation (Fig.6A). The Δ*sucA* strain, lacking an α-ketoglutarate dehydrogenase, showed a slight negative effect on cellulose production, with no significant difference between sorbitol and plain LB media (Fig.6A). In contrast, Δ*sucA* did not change the phenotype with respect to autoaggregation (Fig.6B). Since these mutant strains have an impaired TCA cycle and respiratory pathway, yet some level of cellulose production and autoaggregation is still observed, this could indicate that the TCA cycle is not essential for cellulose production.

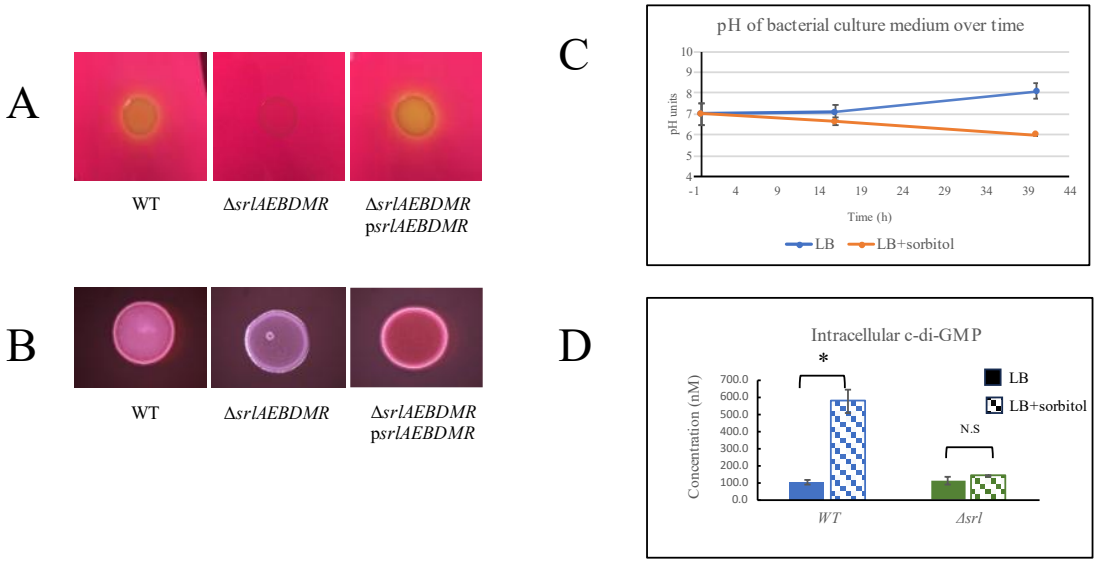

Figure 5. Fermentation is associated with cellulose production in *E. amylovora*. A) Fermentation of sorbitol on sorbitol-MacConkey agar. The yellowish color of colonies indicates an acidic pH from fermentation. B) Cellulose production of Ea1189 WT and *srl* operon mutants on Congo Red amended LB agar with sorbitol. Reddish or pinkish color indicates cellulose production. C) Change in pH of liquid LB growth medium with and without sorbitol over time. D) Intracellular c-di-GMP concentrations measured with HPLC-MS/MS. Error bars signify standard deviation; presence of an asterisk above the bars indicates statistically significant difference observed ($P > 0.05$ by Student's *t* test). Non-significant differences are denoted by N.S.

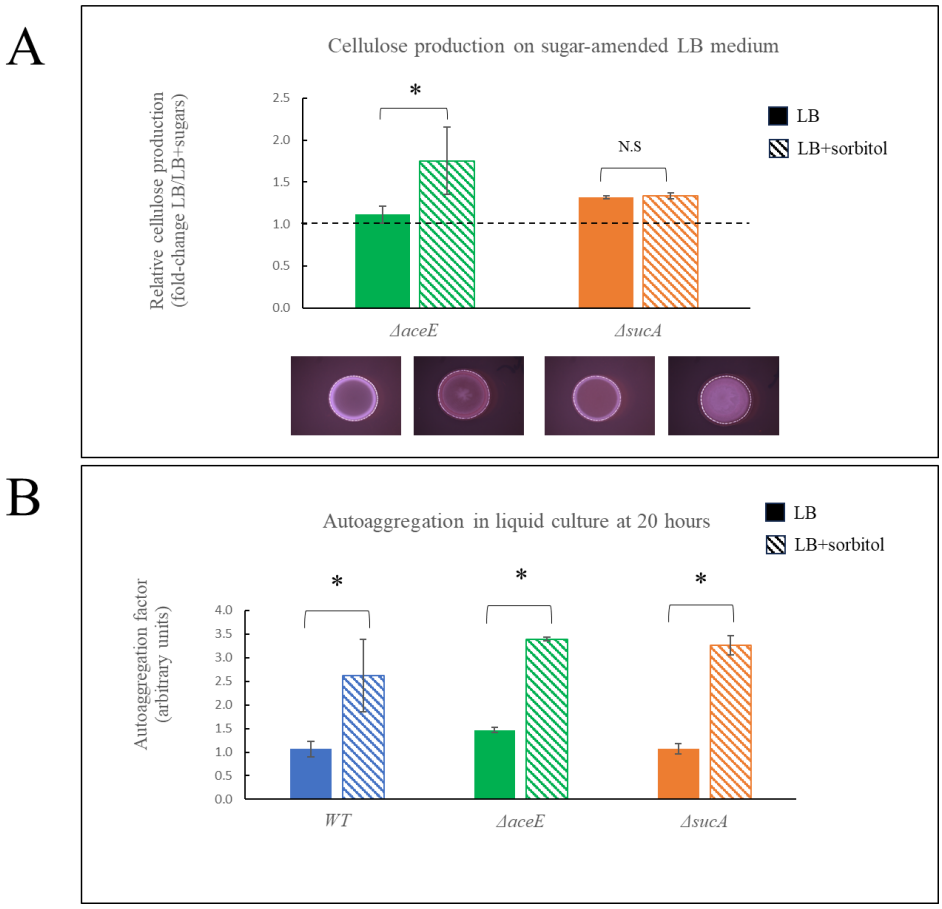

Figure 6. TCA cycle enzymes may not be essential for cellulose production. A) Cellulose production by Ea1189 mutant strains on Congo Red LB agar medium amended with 20 mg/mL sugars. Quantification of macrocolony redness was measured with WT on unamended medium as a reference and denoted by the dotted line. B) Quantification of autoaggregation factor in liquid LB medium amended with sugars after 20 hours of growth. Error bars signify standard deviation; presence of an asterisk above the bars indicates statistically significant difference observed ($P > 0.05$ by Student's *t* test). Non-significant differences are denoted by N.S.

2.5. Discussion

The apple and pear host plants of *E. amylovora* are rich in carbohydrates, mainly sorbitol, sucrose, and small amounts of fructose and glucose. *In vitro* studies of this economically important bacterium often use LB growth medium, which is rich in amino acids and short peptides but does not contain a significant source of carbohydrates. When high levels of host sugars glucose, sucrose, and sorbitol were added to LB medium, we observed visible differences in bacterial growth: in 20 mg/mL sugar treatments, dilution plating revealed CFU/mL were roughly 6-fold lower than unamended LB medium at the sampling time (1.7×10^7 vs. 1.1×10^8 CFU/mL). In addition, the cells were mostly confined to the bottom of the culture tube in visible aggregates, displaying autoaggregation behavior as previously observed in *E. amylovora* (Kharadi & Sundin, 2019). Cellulose production assays revealed that WT Ea1189 produced significantly more cellulose when grown on medium amended with sugars than plain LB, and this phenotype was reflected in liquid media by significantly increased autoaggregation (Fig.1A-B). Interestingly, each sugar produced a very similar phenotype, suggesting that under our experimental conditions there was no specific effect of a certain sugar. The addition of host sugars increased the nutrient density of the growth medium, yet there were far fewer cells of WT Ea1189 in the sugar amended medium than unamended LB at the sampling timepoint. These results seem to suggest some kind of metabolic shift in *E. amylovora* upon sugar treatment, in which energy usage favors biosynthesis of EPS over cell division and proliferation, creating a mass of EPS and relatively few cells. Kharadi & Sundin (2019) discovered similar autoaggregation in a triple PDE mutant which had constitutively high levels of c-di-GMP. The authors noted that cell division, and especially cell separation post division, was impaired in this autoaggregating strain. These findings, in concert with those of this study, could suggest that c-

di-GMP may control carbon metabolism in *E. amylovora*, and vice versa, based on the environmental conditions.

The aggregates produced by WT Ea1189 stained positive for cellulose using Calcoflour white, and were totally degraded by cellulase enzymes on a timescale of hours (Fig.1C-D). These cellulose aggregates displayed interesting physical properties. During the washing stage of these assays, the whitish aggregates appeared very fluffy and fragile, almost cloudlike, and would occasionally break apart into smaller pieces. These smaller pieces were far sturdier and more resistant to shearing even with gentle pipetting and vortexing (data not shown). At no time, however, was any reduction in the aggregate apparent, as cellulose is highly insoluble. These physiochemical properties of cellulose differ greatly from the two other EPSs of *E. amylovora*, amylovoran and levan, which are water soluble, in contrast to cellulose which is water insoluble by definition (Bennett & Billing, 1980; Slack et al., 2017). This basic chemical difference begs the question of whether cellulose plays a different function in the fire blight disease cycle compared with amylovoran and levan. Amylovoran and levan make up a large portion of bacterial ooze, and amylovoran is thought to be constitutively produced throughout the disease cycle, as mutations in the *ams* gene cluster render the bacteria nonpathogenic (Bellemann & Geider, 1992; Slack et al., 2017). These two EPSs also play a role in sliding motility, an important mechanism for bacterial movement in confined spaces, and contribute to biofilm formation (Koczan et al., 2009; Yuan, Eldred, & Sundin, 2022). Cellulose appears to be of more limited use in virulence, to date only observed in xylem tissue with electron microscopy, although tests for the presence of bacterial cellulose in other plant tissues have not been done and would be difficult to perform. Nonetheless, cellulose may be a key EPS for *E. amylovora* to effectively colonize the xylem. Because of its insolubility and ability to resist shear stresses,

cellulose could provide a better matrix to protect bacterial cells from the flow of xylem fluid, where water-soluble amylovoran and levan might be washed away. Furthermore, the ability of cellulose to facilitate cell-cell interaction and autoaggregation may be an important mechanism to keep groups of cells together, where a group would have a higher likelihood of successfully colonizing new areas of the host than a single cell.

Cellulose is an important EPS produced by *E. amylovora* and is under strict regulation by c-di-GMP, a regulator of many virulence factors in this bacterium (Castiblanco & Sundin, 2018; Edmunds et al., 2013). The experiments in this study present evidence that specific enzymes DgcB and PdeC control c-di-GMP levels that influence activity of the *bcs* system, modulating the phenotype of *E. amylovora*. Previous research on this pathogen reveals that although DgcB impacts other virulence factors such as amylovoran production and swimming motility, it exerts a particularly powerful effect on cellulose production. Mutation of this gene alone abolished cellulose production in a transposon mutagenesis study (Yuan, Eldred, Kharadi, et al., 2022), and the results shown here reveal a striking lack of cellulose production even in culture conditions where cellulose production is favored (Fig.3). Furthermore, the expression of the *dgcB* gene has a strong positive effect on cellulose production and caused a distinct increase in autoaggregation in 120 minutes. In sorbitol treated growth medium, intracellular c-di-GMP levels increased in the WT strain, but this increase was not observed in the *dgcB* mutant. This suggests that the effect of sugar amendment on high c-di-GMP phenotypes such as cellulose production and autoaggregation is mediated at least primarily, and potentially exclusively, by DgcB-synthesized c-di-GMP. Bioinformatic analysis of the predicted DgcB protein structure shows, in contrast to many DGCs, that DgcB does not contain a predicted signaling domain; instead, the entire N-terminus is a series of five transmembrane domains followed only by the C-terminal GGDEF

domain (Edmunds et al., 2013). This comparatively simple structure could suggest that DgcB could be regulated more strictly at the transcriptional level, as opposed to the many post-translational mechanisms discovered in other DGCs (Randall et al., 2022). Indeed, mRNA quantification of WT Ea1189 in growth media with and without sorbitol showed an approximately 3-fold increase in *bcsA* and *dgcB* expression in the sorbitol treatment, where cellulose was produced (Fig.4B). In addition, expression of *dgcB* in both growth conditions produced similar levels of cellulose on Congo Red plates (Fig.3A), suggesting that the sorbitol treatment affects transcription of the *dgcB* gene but may not influence protein-level regulation of enzyme activity. However, in liquid medium the Δ8 p*dgcB* strain had a markedly higher autoaggregation factor when sorbitol was added compared to the control condition (Fig.4A). This could be from some unknown effect on protein-level enzyme activity, or it could simply be an effect of abundant carbon substrates facilitating more growth and the biosynthesis of larger cellulose aggregates. While these experiments demonstrate that expression of *dgcB* is likely the most important positive regulator of cellulose production and autoaggregation, it is still unknown what genetic mechanisms facilitate the perception of sugars in the environment and increase transcription of this important gene.

Regardless of enzymatic regulation, part of the pronounced impact of *E. amylovora* DgcB on cellulose could be from membrane localization. In a detailed study of pEtN-cellulose production in *Es. coli*, researchers discovered that DgcC directly interacts with the BcsB subunit of the Bcs complex via the transmembrane domain; a truncated DgcC protein lacking the transmembrane domain failed to produce cellulose (Richter et al., 2020). Since our bioinformatic analysis of *Es. coli* DgcC predicts an identical domain structure and some degree of amino acid sequence similarity to *E. amylovora* DgcB, perhaps a similar mechanism could be at play, where

the membrane localization of DgcB as a part of the Bcs complex gives this DGC such a potent effect on cellulose production. However, it is important to note that the *Es. coli* DgcC activated cellulose production specifically and locally, with no impact on the intracellular levels of c-di-GMP (Richter et al., 2020). This is in contrast to DgcB of *E. amylovora*, mutation of which can change the overall levels of intracellular c-di-GMP, and can have an effect on multiple virulence factors as observed in previous studies (Edmunds et al., 2013; Kharadi et al., 2022b). How single enzymes influence both local and global c-di-GMP signal transduction remains a mysterious research area of molecular biology.

Analysis of the three PDE enzymes of *E. amylovora* revealed PdeC to be the only one affecting cellulose production and autoaggregation under our experimental conditions (Fig.2A). This phenotype is interesting given that comparison of the *pdeC* mutant to *pdeA* and *pdeB* mutants found no change in virulence factors such as biofilm formation, swimming motility, and amylovoran production, even though this gene had the strongest individual impact on c-di-GMP levels (Kharadi et al., 2019b). This suggests that the strong PDE activity of PdeC is directed mostly towards downregulating cellulose. The effect of PdeC was noticeable but less distinct in LB liquid media; the Δ*pdeC* strain produced a visible cellulose-based aggregate even with no sorbitol (Fig2C-E), and the autoaggregation factor was slightly higher, but not statistically significant compared to the wild type (Fig.2B). Mutation of *pdeC* also had no effect on the sorbitol treatments compared to the WT strain (Fig.3A,B). These results in addition to the effects of DgcB may indicate that the stimulation of cellulose production when sorbitol is added is a result of *dgcB* expression and activity, rather than some inactivation or downregulation of PdeC.

Bioinformatic comparison of *E. amylovora* PdeC to *Es. coli* PdeK showed very similar domain structures, and high amino acid sequence homology (Appendix 1). *Es. coli* PdeK is a

known specific regulator of cellulose production and was demonstrated to associate with both *Es. coli* DgcC and the BcsB subunit of the Bcs complex (Richter et al., 2020). PdeC and PdeK also share similar placement in the genome, immediately next to the *bcs* operon (Appendix 1). This comparison, when combined with the experimental data shown here, leads us to propose that PdeC is a specific regulator of cellulose production in *E. amylovora*.

Previous bioinformatic analysis predicts a periplasmic signaling domain between two transmembrane domains at the N-terminus of PdeC and similar proteins such as *Es. coli* PdeK (Kharadi et al., 2019b). Annotated as Gamma-proteobacterial periplasmic signaling domain 3 (GAPES3), the function of this domain and any possible roles in PDE enzyme activity are completely unknown. Truncation of the *pdeC* gene to eliminate the N-terminal GAPES3 domain appeared to abolish PDE activity when assayed for cellulose production (Appendix 2). However, truncated genes can be prone to misfolding, and it should be noted that a transmembrane domain was also eliminated in the truncated version. To more clearly ascertain if the GAPES3 domain is truly necessary for enzymatic activity of PdeC, domain-directed mutagenesis was used to randomly mutate base pairs in the GAPES3 region. A strain was isolated that had a completely intact full-length *pdeC* gene except for 6 point mutations in the target region. This strain also showed a loss of PDE activity when assayed for cellulose production (Appendix 2). This presents strong evidence that the N-terminal GAPES3 domain controls the activity of the active C-terminal EAL domain. We speculate that a post-translational protein-level interaction regulates the activity of PdeC via the GAPES3 domain. While the specific mechanism of signal perception by this domain is still unknown, this information does help explain the surprising finding that the *pdeC* gene was upregulated in response to sorbitol addition (Fig.4B). Since cellulose production is favored under these conditions, and PdeC is a negative regulator of

cellulose production, we expected this gene to be downregulated. However, perhaps inactivation of the enzyme could occur via the GAPES3 domain while allowing the *pdeC* gene to be expressed, allowing *E. amylovora* to have the negative regulator ready for speedy shutoff of cellulose production via protein-level activation of PdeC.

While the precise mechanisms of how addition of host sugars influences c-di-GMP and cellulose via DgcB and PdeC was beyond the scope of this study, the experiments presented here suggest that changes in central carbon metabolism may broadly regulate these processes. The *srl* mutant strain, unable to take up or metabolize sorbitol via glycolysis, failed to ferment sorbitol when this sugar was added to the growth medium (Fig. 5A). This strain showed a concomitant lack of cellulose production and a failure to increase c-di-GMP levels when grown with sorbitol, as in the WT (Fig.5B,D). We found this association between cellulose production and fermentative metabolism interesting given the 6-fold decrease in growth we observed upon addition of sorbitol to LB medium. Glycolysis yields 2 ATP molecules per glucose molecule in addition to a fermentation product such as lactate or acetate, whereas full oxidative metabolism with the Tricarboxylic Acid (TCA) cycle yields up to 31 ATP molecules per glucose molecule (Mookerjee et al., 2017). This supports our data showing slower cell growth associated with less energy-productive glycolysis and fermentation.

Recent research on *E. amylovora* revealed that mutations in *aceE*, a pyruvate dehydrogenase, and *sucA*, an α-ketoglutarate dehydrogenase, impaired the TCA cycle and amylovoran production, with resulting amylovoran levels near the *ams* mutant control (Yuan et al., 2021). We tested these strains for cellulose production and autoaggregation, hypothesizing that if glycolysis and fermentation favor cellulose production, the TCA cycle is less important for this process. The results of these experiments show that, overall, mutation of *aceE* and *sucA*

did not abolish these phenotypes, although the *ΔsucA* strain did show reduced cellulose production on solid medium (Fig.6A,B). While this does potentially support our hypothesis that glycolysis favors cellulose production compared to complete oxidation via the TCA cycle, we do not place too much emphasis on these experiments, as these key metabolic genes may have wide-ranging effects on many cellular processes, and may even be involved in certain fermentation pathways as well (Clark, 1989). A more detailed chemical analysis would likely be necessary to draw conclusions more accurately from this experiment.

Previous chemical analysis of *E. amylovora* have shown that fermentation and oxidation pathways happen simultaneously under ambient oxygen levels, with bacterial cultures producing significant amounts of lactic acid, ethanol, and other organic acids from glycolysis of glucose in addition to CO_2 gas from oxidative metabolism via the TCA cycle (Sutton & Starr, 1959). This study noted that fermentation end products were favored over CO_2 in microaerobic and anaerobic conditions. The effect of producing less energy-efficient fermentative byproducts when nutrients are abundant, even when oxygen is available to facilitate the TCA cycle, is very common in microorganisms and has been called ‘overflow metabolism’ or the Crabtree effect. Overflow metabolism has been studied for decades in the model organism *E. coli*, which ferments glucose to acetic acid when glucose is abundant, even in aerobic conditions (Millard et al., 2021). Thus it is not surprising that *E. amylovora* also ferments carbon substrates in our aerobic *in vitro* experimental conditions. Perhaps having multiple carbon metabolic pathways operating simultaneously allows *E. amylovora* more versatility in deploying different virulence strategies, such as rapid cell proliferation at the stigma tip, or biofilm formation where anabolic biosynthesis pathways are important to produce EPSs, such as cellulose, in addition to cell growth (Kharadi & Sundin, 2021; Koczan et al., 2009; Slack et al., 2022).

Cellulose has been observed microscopically in the xylem of apple tissue, and has been most studied in the context of xylem biofilm formation (Castiblanco & Sundin, 2018; Yuan, Eldred, Kharadi, et al., 2022). The xylem of plants is a confined, watery environment in which oxygen levels can become depleted, as in the case of xylem-limited pathogen *Xylella fastidiosa* (Shriner & Andersen, 2014). In the xylem, fermentation pathways could be favored, facilitating cellulose production, autoaggregation, and biofilm formation in *E. amylovora*. Although xylem fluid is usually considered nutrient-poor, without high carbohydrate levels as studied here, the chemistry of apple xylem is highly variable with seasonal fluctuation of ions, proteins, hormones, amino acids, and some carbohydrates (Biles & Abeles, 1991; Bradfield, 1976; Davison & Young, 1973; Malaguti et al., 2001; Tromp, 1979). Furthermore, infection of some plants by bacterial pathogens can greatly alter soluble carbon allocation and transport, and cell lysis by bacterial pathogens can release additional nutrients (Breia et al., 2021). It is not inconceivable that *E. amylovora* could at certain times encounter sufficient carbohydrates in the xylem tissue for overflow metabolism via fermentation to occur.

In conclusion, we identified a DGC/PDE pair that regulates bacterial cellulose production via c-di-GMP levels; DgcB and PdeC. We discovered that host sugar availability influences *dgcB* expression, causing cellulose production and the associated behavior of autoaggregation. PdeC is a negative regulator of these processes and is at least partially controlled by the N-terminal GAPES3 domain. Sugar fermentation is associated with cellulose production in *E. amylovora* and could favor cellulose production in confined areas such as xylem biofilms, where the physiochemical properties of cellulose may contribute to fire blight disease.

REFERENCES

Abidi, W., Torres-Sánchez, L., Siroy, A., & Krasteva, P. V. (2022). Weaving of bacterial cellulose by the Bcs secretion systems. FEMS Microbiology Reviews, 46(2). https://doi.org/10.1093/femsre/fuab051

Aldridge, P., Metzger, M., & Geider, K. (1997). Genetics of sorbitol metabolism in Erwinia amylovora and its influence on bacterial virulence. Molecular and General Genetics, 256(6). doi: 10.1007/s004380050609.

Almblad, H., Randall, T. E., Liu, F., Leblanc, K., Groves, R. A., Kittichotirat, W., Winsor, G. L., Fournier, N., Au, E., Groizeleau, J., Rich, J. D., Lou, Y., Granton, E., Jennings, L. K., Singletary, L. A., Winstone, T. M. L., Good, N. M., Bumgarner, R. E., Hynes, M. F., Harrison, J. J. (2021). Bacterial cyclic diguanylate signaling networks sense temperature. Nature Communications, 12(1). https://doi.org/10.1038/s41467-021-22176-2

Ausmees, N., Mayer, R., Weinhouse, H., Volman, G., Amikam, D., Benziman, M., & Lindberg, M. (2001). Genetic data indicate that proteins containing the GGDEF domain possess diguanylate cyclase activity. FEMS Microbiology Letters, 204(1), 163–167. https://doi.org/10.1111/j.1574-6968.2001.tb10880.x

Bellemann, P., & Geider, K. (1992). Localization of transposon insertions in pathogenicity mutants of Erwinia amylovora and their biochemical characterization. Journal of General Microbiology,138(5), 931-940.

Bennett, R. A., & Billing, E. (1980). Origin of the Polysaccharide Component of Ooze from Plants Infected with Erwinia amylovora. Journal of General Microbiology, 116(2). https://doi.org/10.1099/00221287-116-2-341

Biles, C. L., & Abeles, F. B. (1991). Xylem sap proteins. Plant Physiology, 96(2). https://doi.org/10.1104/pp.96.2.597

Bobrov, A. G., Kirillina, O., & Perry, R. D. (2005). The phosphodiesterase activity of the HmsP EAL domain is required for negative regulation of biofilm formation in Yersinia pestis. FEMS Microbiology Letters, 247(2), 123–130. https://doi.org/10.1016/j.femsle.2005.04.036

Bogs, J., & Geider, K. (2000). Molecular Analysis of Sucrose Metabolism of Erwinia amylovora and Influence on Bacterial Virulence. Journal of Bacteriology, 182(19). http://www3.ncbi.nlm.nih.gov

Boureau, T., Elmaarouf-Bouteau, H., Garnier, A., Brisset, M.-N., Perino, C., Pucheu, I., & Barny, M.-A. (2006). 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 MPMI, 19(1), 16–24. https://doi.org/10.1094/MPMI

Bradfield, E. G. (1976). Calcium complexes in the xylem sap of apple shoots. Plant and Soil, 44(2). https://doi.org/10.1007/BF00015906

Breia, R., Conde, A., Badim, H., Fortes, A. M., Gerós, H., & Granell, A. (2021). Plant Sweets: From sugar transport to plant–pathogen interaction and more unexpected physiological roles. Plant Physiology, 186(2), 836-852. https://doi.org/10.1093/PLPHYS/KIAB127

Castiblanco, L. F., & Sundin, G. W. (2018). Cellulose production, activated by cyclic di-GMP through BcsA and BcsZ, is a virulence factor and an essential determinant of the three-dimensional architectures of biofilms formed by Erwinia amylovora Ea1189. Molecular Plant Pathology, 19(1), 90–103. https://doi.org/10.1111/mpp.12501

Clark, D. P. (1989). The fermentation pathways of Escherichia coli . FEMS Microbiology Letters, 63(3), 223–234. https://doi.org/10.1111/j.1574-6968.1989.tb03398.x

Datsenko, K. A., & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Biological Sciences, 97(12). https://doi.org/10.1073/pnas.120163297

Davison, R. M., & Young, H. (1973). Abscisic-acid content of xylem sap. Planta, 109(1). https://doi.org/10.1007/BF00385455

Dellagi, A., Brisset, M.-N., Paulin, J.-P., & Expert, D. (1998). Dual Role of Desferrioxamine in Erwinia amylovora Pathogenicity. Molecular Plant-Microbe Interactions MPMI, 11(8).

Edmunds, A. C., Castiblanco, L. F., Sundin, G. W., & Waters, C. M. (2013). Cyclic Di-GMP modulates the disease progression of Erwinia amylovora. Journal of Bacteriology, 195(10), 2155–2165. https://doi.org/10.1128/JB.02068-12

Enomoto, G., Wallner, T., & Wilde, A. (2023). Control of light-dependent behaviour in cyanobacteria by the second messenger cyclic di-GMP. MicroLife, 4. https://doi.org/10.1093/femsml/uqad019

Finn, R. D., Coggill, P., Eberhardt, R. Y., Eddy, S. R., Mistry, J., Mitchell, A. L., Potter, S. C., Punta, M., Qureshi, M., Sangrador-Vegas, A., Salazar, G. A., Tate, J., & Bateman, A. (2016). The Pfam protein families database: Towards a more sustainable future. Nucleic Acids Research, 44(1), 279–285. https://doi.org/10.1093/nar/gkv1344

Galperin, M. Y., Natale, D. A., Aravind, L., & Koonin, E. V. (1999). A specialized version of the HD hydrolase domain implicated in signal transduction. J Mol Microbiol Biotechnol, 1(2):303-5.

Gottesman, S., & Storz, G. (2011). Bacterial small RNA regulators: Versatile roles and rapidly evolving variations. Cold Spring Harbor Perspectives in Biology, 3(12). https://doi.org/10.1101/cshperspect.a003798

Haq, A., & Dawes, E. A. (1971). Pyruvic Acid Metabolism and Ethanol Formation in Erwinia amylovora. Journal of General Microbiology, 68(3). https://doi.org/10.1099/00221287-68-3-295

Hengge, R., Galperin, M. Y., Ghigo, J. M., Gomelsky, M., Green, J., Hughes, K. T., Jenal, U., & Landini, P. (2016). Systematic nomenclature for GGDEF and EAL domaincontaining cyclic di-GMP turnover proteins of Escherichia coli. Journal of Bacteriology, 198(1), 7–11. https://doi.org/10.1128/JB.00424-15

Herbst, S., Lorkowski, M., Sarenko, O., Nguyen, T. K. L., Jaenicke, T., & Hengge, R. (2018). Transmembrane redox control and proteolysis of PdeC, a novel type of c-di- GMP phosphodiesterase. The EMBO Journal, 37(8). https://doi.org/10.15252/embj.201797825

Huang, C. J., Wang, Z. C., Huang, H. Y., Huang, H. Da, & Peng, H. L. (2013). YjcC, a c-di-GMP Phosphodiesterase Protein, Regulates the Oxidative Stress Response and Virulence of Klebsiella pneumoniae CG43. PLoS ONE, 8(7). https://doi.org/10.1371/journal.pone.0066740

Ionescu, M., & Belkin, S. (2009). Overproduction of exopolysaccharides by an Escherichia coli K-12 rpoS mutant in response to osmotic stress. Applied and Environmental Microbiology, 75(2), 483–492. https://doi.org/10.1128/AEM.01616-08

Jahn, C. E., Selimi, D. A., Barak, J. D., & Charkowski, A. O. (2011). The Dickeya dadantii biofilm matrix consists of cellulose nanofibres, and is an emergent property dependent upon the type iii secretion system and the cellulose synthesis operon. Microbiology, 157(10), 2733–2744. https://doi.org/10.1099/mic.0.051003-0

Kharadi, R. R., Castiblanco, L. F., Waters, C. M., & Sundin, G. W. (2019a). Phosphodiesterase genes regulate amylovoran production, biofilm formation, and virulence in Erwinia amylovora. Applied and Environmental Microbiology, 85(1). https://doi.org/10.1128/AEM.02233-18

Kharadi, R. R., Castiblanco, L. F., Waters, C. M., & Sundin, G. W. (2019b). Phosphodiesterase genes regulate amylovoran production, biofilm formation, and virulence in Erwinia amylovora. Applied and Environmental Microbiology, 85(1). https://doi.org/10.1128/AEM.02233-18

Kharadi, R. R., Selbmann, K., & Sundin, G. W. (2022a). A complete twelve-gene deletion null mutant reveals that cyclic di-GMP is a global regulator of phase-transition and host colonization in Erwinia amylovora. PLoS Pathogens, 18(8). https://doi.org/10.1371/journal.ppat.1010737

Kharadi, R. R., Selbmann, K., & Sundin, G. W. (2022b). A complete twelve-gene deletion null mutant reveals that cyclic di-GMP is a global regulator of phase-transition and host colonization in Erwinia amylovora. PLoS Pathogens, 18(8). https://doi.org/10.1371/journal.ppat.1010737

Kharadi, R. R., & Sundin, G. W. (2019). Physiological and microscopic characterization of cyclic-di-GMP-mediated autoaggregation in Erwinia amylovora. Frontiers in Microbiology, 10. https://doi.org/10.3389/fmicb.2019.00468

Kharadi, R. R., & Sundin, G. W. (2021). Dissecting the process of xylem colonization through biofilm formation in Erwinia amylovora. In Journal of Plant Pathology, 103, 41–49. https://doi.org/10.1007/s42161-020-00635-x

Koczan, J. M., McGrath, M. J., Zhao, Y., & Sundin, G. W. (2009). Contribution of Erwinia amylovora exopolysaccharides amylovoran and levan to biofilm formation: Implications in pathogenicity. Phytopathology, 99(11), 1237–1244. https://doi.org/10.1094/PHYTO-99-11-1237

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. Methods, 25(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Malaguti, D., Millard, P., Wendler, R., Hepburn, A., & Tagliavini, M. (2001). Translocation of amino acids in the xylem of apple (Malus domestica Borkh.) trees in spring as a consequence of both N remobilization and root uptake. Journal of Experimental Botany, 52(361). https://doi.org/10.1093/jxb/52.361.1665

Mansfield, J., Genin, S., Magori, S., Citovsky, V., Sriariyanum, M., Ronald, P., Dow, M., Verdier, V., Beer, S. V., Machado, M. A., Toth, I., Salmond, G., & Foster, G. D. (2012). Top 10 plant pathogenic bacteria in molecular plant pathology. Molecular Plant Pathology, 13(6), 614–629. https://doi.org/10.1111/j.1364-3703.2012.00804.x

Massie, J. P., Reynolds, E. L., Koestler, B. J., Cong, J. P., Agostoni, M., & Waters, C. M. (2012). Quantification of high-specificity cyclic diguanylate signaling. Proceedings of the National Academy of Sciences of the United States of America, 109(31), 12746–12751. https://doi.org/10.1073/pnas.1115663109

Millard, P., Enjalbert, B., Uttenweiler-Joseph, S., Portais, J. C., & Létisse, F. (2021). Control and regulation of acetate overflow in Escherichia coli. ELife, 10. https://doi.org/10.7554/eLife.63661

Mohite, B. V., & Patil, S. V. (2014). A novel biomaterial: Bacterial cellulose and its new era applications. Biotechnology and Applied Biochemistry, 61(2), 101–110. https://doi.org/10.1002/bab.1148

Mookerjee, S. A., Gerencser, A. A., Nicholls, D. G., & Brand, M. D. (2017). Quantifying intracellular rates of glycolytic and oxidative ATP production and consumption using extracellular flux measurements. Journal of Biological Chemistry, 292(17), 7189–7207. https://doi.org/10.1074/jbc.M116.774471

Morgan, J. L. W., McNamara, J. T., & Zimmer, J. (2014). Mechanism of activation of bacterial cellulose synthase by cyclic di-GMP. Nature Structural and Molecular Biology, 21(5), 489–496. https://doi.org/10.1038/nsmb.2803

Naomi, R., Idrus, R. B. H., & Fauzi, M. B. (2020). Plant-vs. Bacterial-derived cellulose for wound healing: A review. International Journal of Environmental Research and Public Health, 17(18), 1–25. https://doi.org/10.3390/ijerph17186803

Norelli, J. L., Jones, A. L., & Aldwinckle, H. S. (2003). Fire Blight Management in the Twenty-first Century Using New Technologies that Enhance Host Resistance in Apple. Plant Disease, 87(7).

Piqué, N., Miñana-Galbis, D., Merino, S., & Tomás, J. M. (2015). Virulence factors of Erwinia amylovora: A review. International Journal of Molecular Sciences, 16(6), 12836–12854. https://doi.org/10.3390/ijms160612836

Pusey, P. L., Rudell, D. R., Curry, E. A., & Mattheis, J. P. (2008). Characterization of Stigma Exudates in Aqueous Extracts from Apple and Pear Flowers. Hortscience, 43(5).

Randall, T. E., Eckartt, K., Kakumanu, S., Price-Whelan, A., Dietrich, L. E. P., & Harrison, J. J. (2022). Sensory Perception in Bacterial Cyclic Diguanylate Signal Transduction. In Journal of Bacteriology (Vol. 204, Issue 2). https://doi.org/10.1128/jb.00433-21

Reinders, A., Hee, C. S., Ozaki, S., Mazur, A., Boehm, A., Schirmer, T., & Jenal, U. (2016). Expression and genetic activation of cyclic di-GMP-specific phosphodiesterases in Escherichia coli. Journal of Bacteriology, 198(3), 448–462. https://doi.org/10.1128/JB.00604-15

Richter, A. M., Possling, A., Malysheva, N., Yousef, K. P., Herbst, S., von Kleist, M., & Hengge, R. (2020). Local c-di-GMP Signaling in the Control of Synthesis of the E. coli Biofilm Exopolysaccharide pEtN-Cellulose. Journal of Molecular Biology, 432(16), 4576–4595. https://doi.org/10.1016/j.jmb.2020.06.006

Römling, U., & Galperin, M. Y. (2015). Bacterial cellulose biosynthesis: Diversity of operons, subunits, products, and functions. Trends in Microbiology, 23(9), 545–557. https://doi.org/10.1016/j.tim.2015.05.005

Römling, U., Galperin, M. Y., & Gomelsky, M. (2013). Cyclic di-GMP: the First 25 Years of a Universal Bacterial Second Messenger. Microbiology and Molecular Biology Reviews, 77(1), 1–52. https://doi.org/10.1128/mmbr.00043-12

Ross, P., Weinhouse, H., Aloni, Y., Michaeli, D., Weinberger-Ohana, P., Mayer, R., Braun, S., de Vroomt, E., van der Marelt, G. A., van Boomt, J. H., & Benziman, M. (1973). Regulation of cellulose synthesis in Acetobacter xylinum by cyclic diguanylic acid. M. J. PhysioL, 232(6).

Rossi, C. S., Barrientos-Moreno, L., Paone, A., Cutruzzolà, F., Paiardini, A., Espinosa-Urgel, M., & Rinaldo, S. (2022). Nutrient Sensing and Biofilm Modulation: The Example of L-arginine in Pseudomonas. International Journal of Molecular Sciences, 23(8). https://doi.org/10.3390/ijms23084386

Ryjenkov, D. A., Simm, R., Römling, U., & Gomelsky, M. (2006). The PilZ domain is a receptor for the second messenger c-di-GMP: The PilZ domain protein YcgR controls motility in enterobacteria. Journal of Biological Chemistry, 281(41), 30310–30314. https://doi.org/10.1074/jbc.C600179200

Salomone-Stagni, M., Bartho, J. D., Kalita, E., Rejzek, M., Field, R. A., Bellini, D., Walsh, M. A., & Benini, S. (2018). Structural and functional analysis of Erwinia amylovora SrlD. The first crystal structure of a sorbitol-6-phosphate 2-dehydrogenase. Journal of Structural Biology, 203(2), 109–119. https://doi.org/10.1016/j.jsb.2018.03.010

Schachterle, J. K., Onsay, D. M., & Sundin, G. W. (2019). Small RNA ArcZ Regulates Oxidative Stress Response Genes and Regulons in Erwinia amylovora. Frontiers in Microbiology, 10. https://doi.org/10.3389/fmicb.2019.02775

Schachterle, J. K., Zeng, Q., & Sundin, G. W. (2019). Three Hfq-dependent small RNAs regulate flagellar motility in the fire blight pathogen Erwinia amylovora. Molecular Microbiology, 111(6), 1476–1492. https://doi.org/10.1111/mmi.14232

Sebaihia, M., Bocsanczy, A. M., Biehl, B. S., Quail, M. A., Perna, N. T., Glasner, J. D., DeClerck, G. A., Cartinhour, S., Schneider, D. J., Bentley, S. D., Parkhill, J., & Beer, S. V. (2010). Complete genome sequence of the plant pathogen Erwinia amylovora strain ATCC 49946. Journal of Bacteriology, 192(7), 2020–2021. https://doi.org/10.1128/JB.00022-10

Serra, D. O., Richter, A. M., & Hengge, R. (2013). Cellulose as an architectural element in spatially structured escherichia coli biofilms. Journal of Bacteriology, 195(24), 5540–5554. https://doi.org/10.1128/JB.00946-13

Shriner, A. D., & Andersen, P. C. (2014). Effect of Oxygen on the Growth and Biofilm Formation of Xylella fastidiosa in Liquid Media. Current Microbiology, 69(6), 866–873. https://doi.org/10.1007/s00284-014-0660-2

Slack, S. M., Schachterle, J. K., Sweeney, E. M., Kharadi, R. R., Peng, J., Botti-Marino, M., Bardaji, L., Pochubay, E. A., & Sundin, G. W. (2022). In-Orchard Population Dynamics of Erwinia amylovora on Apple Flower Stigmas. Phytopathology, 112(6), 1214–1225. https://doi.org/10.1094/PHYTO-01-21-0018-R

Slack, S. M., Zeng, Q., Outwater, C. A., & Sundin, G. W. (2017). Microbiological examination of erwinia amylovora exopolysaccharide ooze. Phytopathology, 107(4), 403–411. https://doi.org/10.1094/PHYTO-09-16-0352-R

Sutton, D. D., & Starr, M. P. (1959). Anaerobic dissimilation of glucose by Erwinia amylovora. Journal of Bacteriology, 78(3), 427-31. https://journals.asm.org/journal/jb

Takle, G. W., Toth, I. K., & Brurberg, M. B. (2007). Evaluation of reference genes for real-time RT-PCR expression studies in the plant pathogen Pectobacterium atrosepticum. BMC Plant Biology, 7. https://doi.org/10.1186/1471-2229-7-50

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. Molecular Biology and Evolution, 30(12), 2725–2729. https://doi.org/10.1093/molbev/mst197

Tarutina, M., Ryjenkov, D. A., & Gomelsky, M. (2006). An unorthodox bacteriophytochrome from Rhodobacter sphaeroides involved in turnover of the second messenger c-di-GMP. Journal of Biological Chemistry, 281(46), 34751–34758. https://doi.org/10.1074/jbc.M604819200

Tromp, J. (1979). Seasonal Variations in the Composition of Xylem Sap of Apple with Respect to K, Ca, Mg, and N. Zeitschrift Für Pflanzenphysiologie, 94(3). https://doi.org/10.1016/s0044-328x(79)80157-9

Waldron, E. J., Snyder, D., Fernandez, N. L., Sileo, E., Inoyama, D., Freundlich, J. S., Waters, C. M., Cooper, V. S., & Neiditch, M. B. (2019). Structural basis of DSF recognition by its receptor rpfr and its regulatory interaction with the DSF synthase RpfF. PLoS Biology, 17(2). https://doi.org/10.1371/journal.pbio.3000123

Wang, D., Korban, S. S., & Zhao, Y. (2009). The Rcs phosphorelay system is essential for pathogenicity in Erwinia amylovora. Molecular Plant Pathology, 10(2), 277–290. https://doi.org/10.1111/j.1364-3703.2008.00531.x

Wang, Z., & Stutte, G. W. (1992). The Role of Carbohydrates in Active Osmotic Adjustment in Apple under Water Stress. J. AMER. SOC. HORT. SCI, 117(5).

Yap, M. N., Yang, C. H., Barak, J. D., Jahn, C. E., & Charkowski, A. O. (2005). The Erwinia chrysanthemi type III secretion system is required for multicellular behavior. Journal of Bacteriology, 187(2), 639–648. https://doi.org/10.1128/JB.187.2.639-648.2005

Yoo, B. K., & Chen, J. (2010). Role of Cellulose in Protecting Shiga Toxin-Producing Escherichia coli against Osmotic and Chlorine Stress. J Food Pro, 73(11), 2084-2088. doi: 10.4315/0362-028x-73.11.2084.

Yuan, X., Eldred, L. I., Kharadi, R. R., Slack, S. M., & Sundin, G. W. (2022). The RNA-Binding Protein ProQ Impacts Exopolysaccharide Biosynthesis and Second Messenger Cyclic di-GMP Signaling in the Fire Blight Pathogen Erwinia amylovora. Applied and Environmental Microbiology, 88(9). https://doi.org/10.1128/aem.00239-22

Yuan, X., Eldred, L. I., & Sundin, G. W. (2022). Exopolysaccharides amylovoran and levan contribute to sliding motility in the fire blight pathogen Erwinia amylovora. Environmental Microbiology, 24(10), 4738–4754. https://doi.org/10.1111/1462-2920.16193

Yuan, X., McGhee, G. C., Slack, S. M., & Sundin, G. W. (2021). A novel signaling pathway connects thiamine biosynthesis, bacterial respiration, and production of the exopolysaccharide amylovoran in Erwinia amylovora. Molecular Plant-Microbe Interactions, 34(10). https://doi.org/10.1094/MPMI-04-21-0095-R

Zeng, Q., McNally, R. R., & Sundin, G. W. (2013). Global small RNA chaperone Hfq and regulatory small RNAs are important virulence regulators in erwinia amylovora. Journal of Bacteriology, 195(8), 1706–1717. https://doi.org/10.1128/JB.02056-12

Zeng, Q., & Sundin, G. W. (2014). Genome-wide identification of Hfq-regulated small RNAs in the fire blight pathogen Erwinia amylovora discovered small RNAs with virulence regulatory function. BMC Genomics, 15(1), 414. doi: 10.1186/1471-2164-15-414.

Zhao, Y., Sundin, G. W., & Wang, D. (2009). Construction and analysis of pathogenicity Island deletion mutants of Erwinia amylovora. Canadian Journal of Microbiology, 55(4), 457–464. https://doi.org/10.1139/W08-147

Zhao, Y., Wang, D., Nakka, S., Sundin, G. W., & Korban, S. S. (2009). Systems level analysis of two-component signal transduction systems in Erwinia amylovora: Role in virulence, regulation of amylovoran biosynthesis and swarming motility. BMC Genomics, 10. https://doi.org/10.1186/1471-2164-10-245

APPENDIX A: BIOINFORMATIC OBSERVATIONS

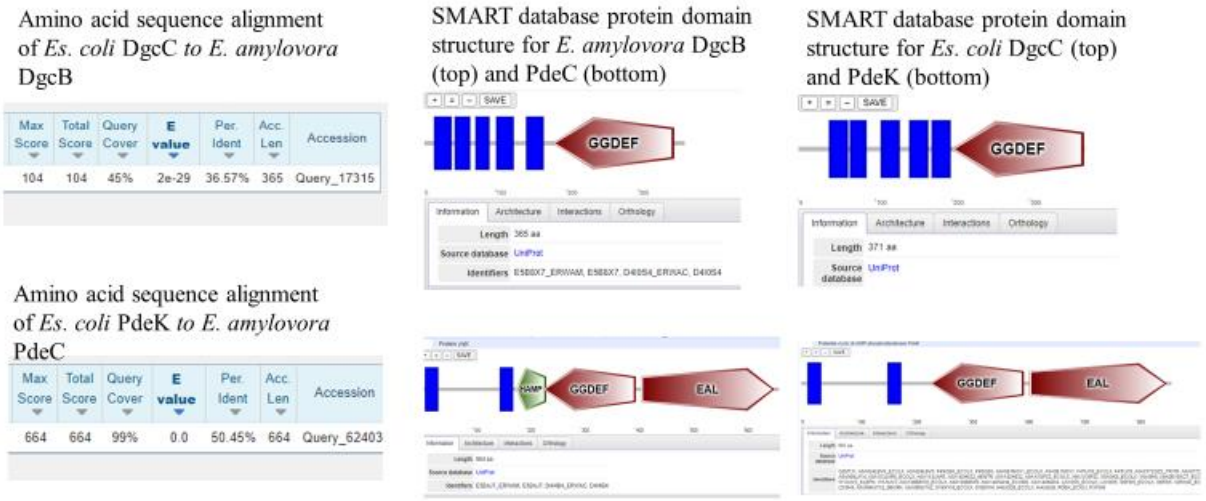

Figure A1. Screenshot summary of BLAST and SMART database comparison of amino acid sequences of *Es. coli* DgcC and PdeK and *E. amylovora* DgcB and PdeC.

In the model organism *Es. coli*, production of a specific EPS pEtN-cellulose is regulated specifically by the DGC DgcC and PDE PdeK. This comparison of the amino acid sequence and domain structure was conducted to evaluate any potential similarities between these enzymes and the *E. amylovora* enzymes experimentally demonstrated to regulate cellulose production, DgcB and PdeC. The PDEs appear very similar to each other in domain structure and sequence similarity, whereas the DGCs had less percent identity in the amino acid sequence but still showed the same domain structure.

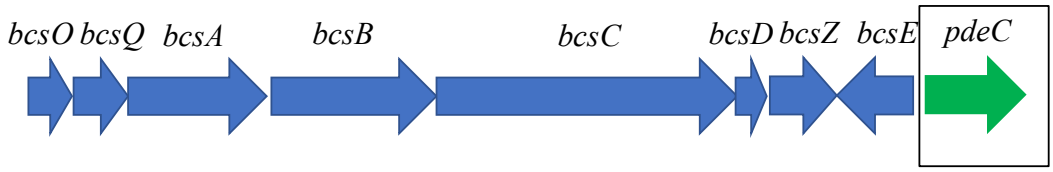

Figure A2. Location of the *pdeC* gene next to the *bcs* operon in *E. amylovora*. Regulatory genes are often encoded close to the genes they regulate; the location of the *pdeC* gene immediately next to the bcs operon is evidence of the importance of *pdeC* as a regulator of cellulose in *E. amylovora*.

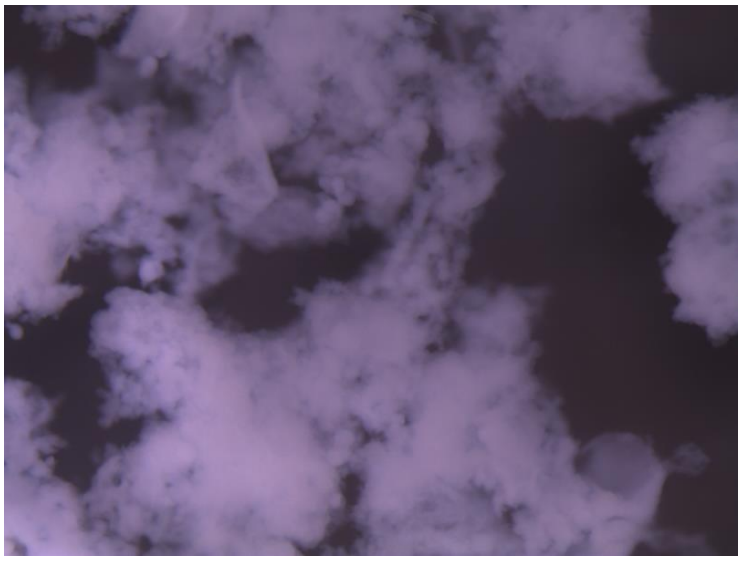

Figure A3. Image at 30x magnification of washed bacterial cellulose produced by Ea1189 against a dark background for contrast. The fluffy, cloudlike aggregates showed affinity for each other, often combining with each other when gently stirred, and appeared to be resistance to dissolution in water, growth medium, or phosphate buffer solutions. Cellulose also collected in the bottom of a centrifuge tube after centrifugation, unlike amylovoran, which remains in the supernatant as evidenced by the amylovoran assay used in previous studies (Kharadi et al., 2019). We make the simple observation that the insolubility of cellulose compared to other EPSs could give this EPS a unique role in virulence, such as protecting cells from the flow of xylem fluid.

APPENDIX B: DOMAIN-DIRECTED MUTAGENESIS

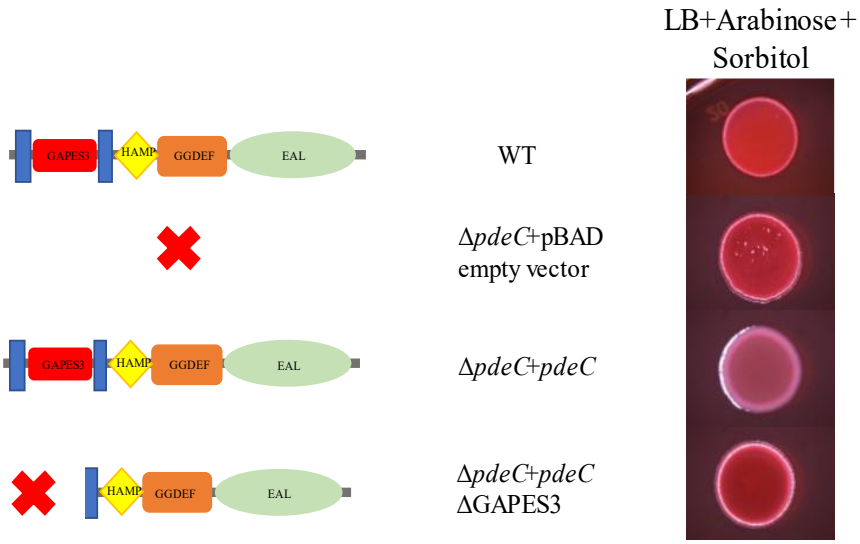

Figure B1. Truncation of the *pdeC* gene on a plasmid vector abolishes PDE activity.

Because the experiments in this study demonstrated that PdeC negatively regulates cellulose production in *E. amylovora*, and because many PDE enzymes are in turn regulated by N-terminal signaling domains, we investigated whether the GAPES3 domain could alter PdeC enzymatic activity. The function of the GAPES3 domain is previously unknown. We sought to create a strain with a truncated gene that would eliminate the region containing this signaling domain. We hypothesized that this truncation would greatly increase or decrease PdeC activity, which could then be visualized by testing for cellulose production.

A truncated *pdeC* gene was designed by designing primers to start after the N-terminal GAPES3 domain region of the *pdeC* gene and insert a start codon, creating a shortened *pdeC* gene with a start codon that was cloned into an arabinose-inducible plasmid vector. Graphics on the left depict the domain structure of the PdeC protein, with red X's indicate the missing gene or domain in the plasmid. The empty vector, full length gene, and truncated gene were cloned into the Δ*pdeC* strain and tested for cellulose production. While complementation of the full length *pdeC* gene decreased cellulose production, as observed in other experiments in this study, complementation with the truncated version missing the GAPES3 domain did not appear to alter the cellulose phenotype, indicating that the GAPES3 domain could be critical to PDE function.

Note that one of the transmembrane domains (noted in blue) was also deleted in this process, potentially affecting membrane localization. This limits the quality of the results, as truncated proteins may not fold correctly, be degraded prematurely, or not be transported to the correct location of the cell. The rest of the protein was intact, including the EAL active site, as

Figure B1. (cont'd)
confirmed by Sanger sequencing. These interesting results, despite potential drawbacks, inspired us to try a different approach to verifying if the GAPES3 is essential for PdeC activity. The technique chosen was domain-directed mutagenesis, where error-prone DNA polymerase randomly mutagenizes only a select region of the gene. This maintains the gene at exactly the same length and maintains the sequence and location of the transmembrane domains and the rest of the gene except the GAPES3 domain, eliminating the drawbacks of the previous experiment.

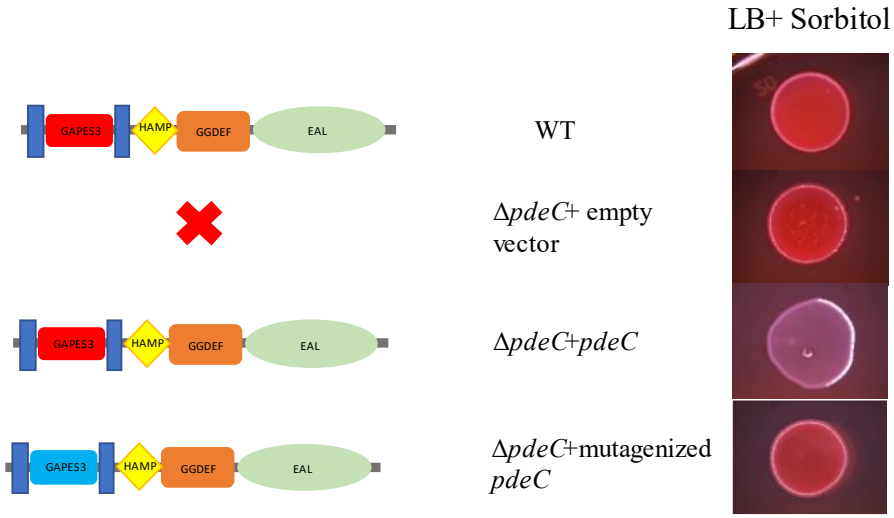

Figure B2. Domain directed mutagenesis resulted in a mutagenized GAPES3 domain with a fully intact gene – mutagenized domain denoted in blue.

Domain-directed mutagenesis was performed on the GAPES3 domain of *pdeC* using the EZ Clone GeneMorph II Domain Mutagenesis Kit (Agilent) according to manufacturer instructions. In brief, error-prone polymerases were used to amplify the GAPES3 region of the *pdeC* gene, and the product of this PCR was used as a megaprimer to resynthesize the pBBR1-MCS5::*pdeC* plasmid, with the result of an intact plasmid and full-length *pdeC* gene with a randomly mutagenized GAPES3 domain. Results were analyzed via Sanger sequencing.

Mutant plasmids from PCR based mutagenesis were screened on Congo Red amended medium for increased cellulose production, and the results were confirmed with Sanger sequencing. 600 colonies were screened, 11 of which showed enhanced cellulose production, indicating a lack of PdeC activity. Of these 11, 10 were either frame shift or nonsense mutations which altered the sequence of the entire gene. The selected strain showed a lack of PdeC activity as determined by a cellulose assay. The strain shown possessed a completely intact *pdeC* gene except for 6 missense mutations in the GAPES3 domain, showing that this signaling domain controls enzymatic activity of the EAL domain.

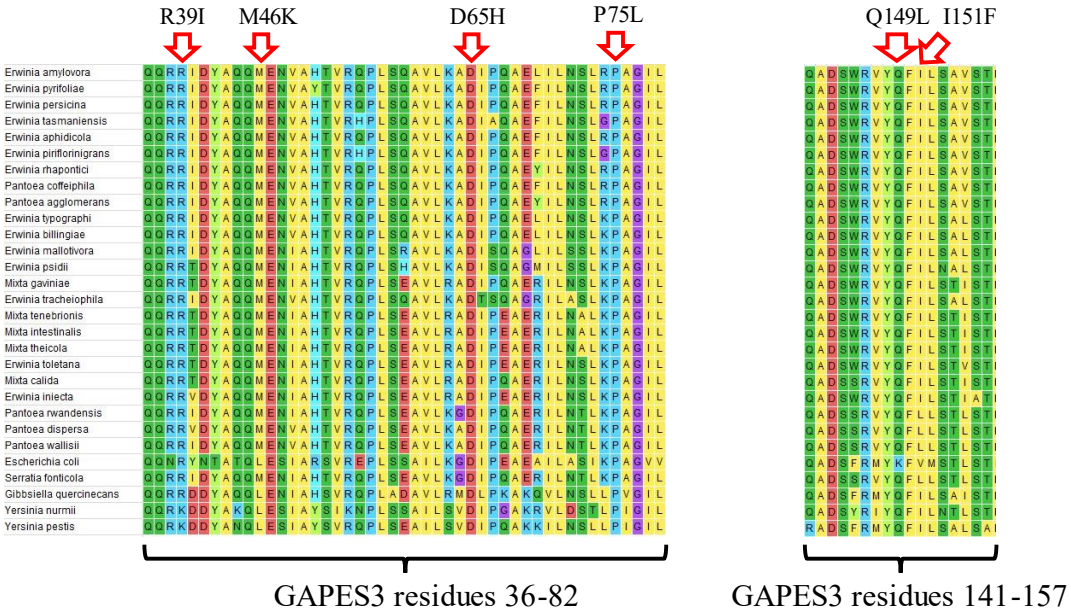

Figure B3. Domain directed mutagenesis caused six amino acid changes in the GAPES3 domain. Labeled arrows show the location and amino acid changes that occurred. Based on cellulose assays, these residues appear to be critical to the function of PdeC.

The intriguing results from the domain directed mutagenesis experiment prompted us to examine the importance of the 6 amino acid residues that were changed. We aligned the GAPES3 domain amino acid sequence of *E. amylovora* to those from other related organisms.

Amino acid sequences from 29 bacterial GAPES3 domains were aligned as shown, showing the high homology of these regions, especially in the amino acids that were randomly mutated. This is especially interesting, as the conservation of these residues provides further evidence that they are essential to proper protein function. In addition, this analysis reveals the conservation of the GAPES3 domain overall. Perhaps this unknown signaling domain plays a similar role in a wide variety of organisms.

APPENDIX C: AMINO ACID SCREENING

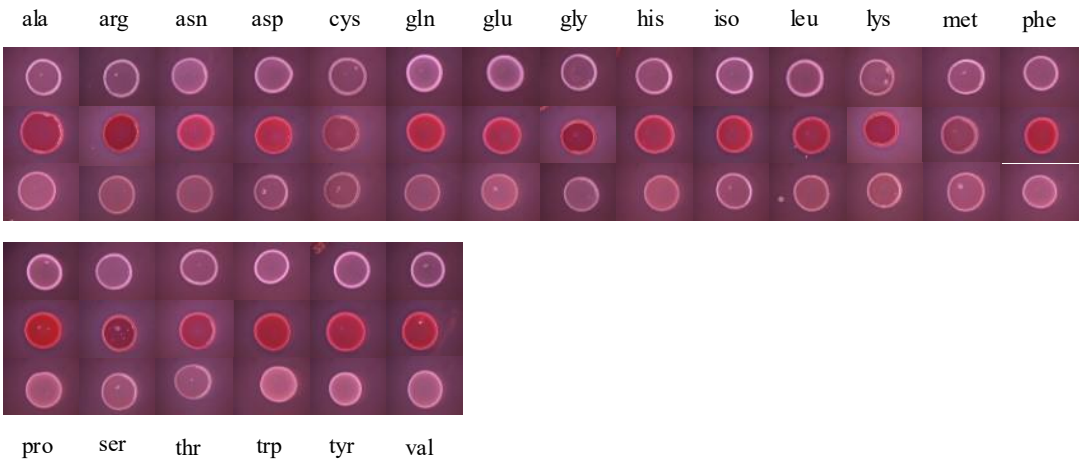

Figure C1. Cellulose production on MBMA minimal medium with sorbitol and 2 mM selected amino acids. Top row: WT. Middle row: *pdeC* mutant. Bottom row: *bcsA* mutant.

WT Ea1189 produces cellulose on LB agar when amended with sorbitol, but not on plain LB medium. We tested a minimal medium (MBMA) containing the same (20mg/mL) amount of sorbitol, and found that the WT strain also failed to produce cellulose. Since LB medium is composed mainly of amino acids, we hypothesized that the combination of sugars and amino acids is necessary for the production of cellulose.

In addition, since the signal the GAPES3 domain of PdeC is unknown, we hypothesized that the availability of one or several amino acids influences the activity of PdeC. 2 mM concentrations

Figure C1. (cont'd)

of the 20 proteinogenic amino acids were added to MBMA agar plates amended with Congo Red dye and E. amylovora strains were tested for cellulose production.

Little or no effect was observed from the amino acid additions on the WT or *bcsA* mutant. However, the *pdeC* mutant showed some striking differences, particularly methionine (met), which produced less cellulose. This could suggest that the availability of amino acids impacts *dgcB* expression, but the results are inconclusive. It does not appear that individual free amino acids alter the activity of *pdeC*.

This experiment was not continued because none of the amino acids appeared to have an effect on the WT strain cellulose production. While the methionine results on the pdeC mutant strain certainly do appear interesting, addition of methionine to liquid LB and MBMA media did not produce a consistently different phenotype from the control (data not shown). Further experiments in this direction were not conducted.

The signal that impacts the periplasmic GAPES3 domain of PdeC remains to be discovered and would present a fascinating future research topic with implications in plant pathology and molecular signal transduction.