A Novel Signaling Pathway Connects Thiamine Biosynthesis, Bacterial Respiration, and Production of the Exopolysaccharide Amylovoran in *Erwinia amylovora*

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Erwinia amylovora is a plant pathogen causing necrotrophic fire blight disease of apple, pear, and other rosaceous plants. This bacterium colonizes host vascular tissues via the production of exopolysaccharides (EPSs) including amylovoran. It is well-established that the nearly ubiquitous plasmid pEA29 of *E. amylovora* is an essential virulence factor, but the underlying mechanism remains uncharacterized. Here, we demonstrated that pEA29 was required for *E. amylovora* to produce amylovoran and to form a biofilm, and this regulation was dependent on the thiamine biosynthesis operon *thiOSGF*. We then conducted carbohydrate and genetic analyses demonstrating that the thiamine-mediated effect on amylovoran production was indirect, as cells lacking *thiOSGF* produced an EPS that did not contain glucuronic acid, one of the key components of amylovoran, whereas the transcriptional activity and RNA levels of the amylovoran biosynthesis genes were not altered. Alternatively, addition of exogenous thiamine restored amylovoran production in the pEA29-cured strain of *E. amylovora* and positively impacted amylovoran production in a dose-dependent manner. Individual deletion of several chromosomal thiamine biosynthesis genes also affected amylovoran production, implying that a complete thiamine biosynthesis pathway is required for the thiamine-mediated effect on amylovoran production in *E. amylovora*. Finally, we determined that an imbalanced tricarboxylic acid cycle negatively affected amylovoran production, which was restored by addition of exogenous thiamine or overexpression of the *thiOSGF* operon. In summary, our report revealed a novel signaling pathway that impacts *E. amylovora* virulence in which thiamine biosynthesis

enhances bacterial respiration that provides energetic requirements for the biosynthesis of EPS amylovoran.

Keywords: amylovoran, bacterial respiration, fire blight, pEA29, pEA29 plasmid, thiamine biosynthesis

Erwinia amylovora is a phytopathogenic enterobacterium that causes the disease fire blight in rosaceous plants such as apples and pears (Griffith et al. 2003; Malnoy et al. 2012). Pathogenesis of *E. amylovora* is dependent upon the expression of a type III secretion system (T3SS), translocation to plants of the major T3SS effector DspA/E, and production of the exopolysaccharide (EPS) amylovoran (Malnoy et al. 2012; Oh and Beer 2005; Piqué et al. 2015; Yuan et al. 2021). This bacterium can form biofilms in host vascular tissues via the production and secretion of EPSs, resulting in disruption of the water flow and the shepherd's crook wilting symptom characteristic of fire blight (Geider 2000; Koczan et al. 2009; Sjulín and Beer 1978). Amylovoran is the most important EPS produced by *E. amylovora* and is a pathogenicity factor, as *E. amylovora* strains that are unable to synthesize amylovoran are not pathogenic (Bellemann and Geider 1992). In addition to amylovoran, EPSs, including levan and cellulose, are also involved in *E. amylovora* biofilm formation in vitro and in planta, and the absence of either of these EPSs resulted in reduced disease symptoms of fire blight in host plants (Castiblanco and Sundin 2018; Koczan et al. 2009).

Amylovoran is a high molecular-weight acidic heteropolymer composed of a repeating unit containing three galactose residues and a sidechain of glucuronic acid, galactose, and pyruvate, and 10% of the core α -galactose is linked to a glucose residue (Nimtz et al. 1996). Early studies have shown that the amylovoran biosynthetic enzymes are encoded within a 12-gene operon (*ams* operon) located in the *E. amylovora* chromosome (Bugert and Geider 1995). Mutational analysis of all 12 genes of the *ams* operon resulted in no amylovoran production and the respective mutants failed to induce fire blight, suggesting that all genes are required for amylovoran biosynthesis (Bellemann and Geider 1992; Bugert and Geider 1995; Menggad and Laurent 1998). The functions of the *ams* gene products are thought to be involved in the assembly of amylovoran from uridine diphosphate (UDP)-sugar precursors, including UDP-glucose, UDP-galactose, and UDP-glucuronic acid, and in the transport of amylovoran through cell membranes (Geider 2000). Since the first discovery of amylovoran in the 1980s, the regulation of the *ams* operon continues to be enigmatic. Two transcriptional regulators, RcsA and RcsB, have been shown to control the expression of *ams*

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genes by binding to the promoter region of the *ams* operon (Bereswill and Geider 1997; Kelm et al. 1997). Bacterial nucleotide second messengers, including c-di-GMP (bis-[3' to 5']-cyclic dimeric guanosine monophosphate) and (p)ppGpp (guanosine tetraphosphate or pentaphosphate), also modulate amylovoran production in *E. amylovora* (Edmunds et al. 2013; Yang et al. 2020); however, the mechanisms are still undetermined.

Almost all *E. amylovora* strains contain the nonconjugative plasmid pEA29, which varies in size from approximately 28 to 32 kb; the importance of this plasmid to fire blight infection was confirmed by independent studies from the 1980s indicating a delay in the onset of symptoms produced by pEA29-cured strains (Falkenstein et al. 1989; Laurent et al. 1989). Similar pEA29-like plasmids are conserved in enterobacterial pathogens related to *E. amylovora*, including *E. pyrifoliae* isolated in Korea, *Erwinia* spp. causing bacterial shoot blight of pear in Japan, and *E. piriflorinigrans*, a pathogen causing necrosis of pear flowers in Spain (Barbé et al. 2013; Maxson-Stein et al. 2003). All of these pEA29-related plasmids exhibit considerable synteny and conservation of putative virulence genes such as the *thiOSGF* operon, encoding components of a thiamine biosynthesis system (McGhee and Jones 2000).

Plasmids are well-known to encode virulence factors in bacterial pathogens of plants and animals (Pilla and Tang 2018; Sundin 2007). For example, a study conducted in the bean pathogen *Pseudomonas syringae* pv. *phaseolicola* demonstrated that curing of the 154-kb indigenous plasmid pAV511 resulted in a loss of virulence on bean (Jackson et al. 1999). *Pantoea agglomerans* pv. *gypsophylae* (formerly known as *E. herbicola* pv. *gypsophylae*) elicits gall formation on gypsophila and relies on a plasmid-encoded T3SS (Nissan et al. 2019). In human-pathogenic bacteria, particularly members of family Enterobacteriaceae, there have been more studies corroborating plasmid-mediated virulence regulation (Pilla and Tang 2018). For example, plasmid pSLT2 of *Salmonella enterica* encodes fimbrial structures that are necessary for adhesion of bacteria to host tissues (Bäumler et al. 1996; Rotger and Casadesús 1999).

Different from the above-mentioned plasmids, independent sequence analyses of pEA29 from *E. amylovora* strains Ea88 and CFBP1430 have shown that no known pathogenicity or virulence genes are present in this plasmid (McGhee and Jones 2000; Smits et al. 2010b). At least 33 open reading frames (ORFs) were annotated, including thiamine biosynthesis genes *thiO*, *thiS*, *thiG*, and *thiF*, a methionine sulfoxide reductase encoding gene *msrA*, a nucleoid-structuring protein-encoding gene *hns*, partitioning protein genes *parA* and *parB*, and genes that are associated with chemotaxis, choline transport, transcriptional regulation, toxin-antitoxin systems, and unknown functions (McGhee and Jones 2000; Smits et al. 2010b).

Previously, we conducted a gain-of-function assay in the *E. amylovora* strain Ea110 showing that complementation of the pEA29 *thiOSGF* operon alone could restore the reduced virulence phenotype of a pEA29-cured strain in immature pears (McGhee and Sundin 2008), but the functional mechanism of thiamine-mediated complementation remains unknown. Thiamine, or vitamin B₁, is an essential component of mammalian diets and is synthesized in plants, fungi, and bacteria (Manzetti et al. 2014). In addition, thiamine pyrophosphate (TPP), an active form of thiamine, serves as an essential cofactor of several enzymes involved primarily in carbohydrate catabolism including glycolysis, the tricarboxylic acid (TCA) cycle, and the pentose phosphate pathway (Dunn 1998; Sudarsan et al. 2005). In light of the importance of thiamine to the energy status of bacterial cells, we hypothesized that thiamine biosynthesis is a critical requirement needed for EPS amylovoran production in *Erwinia* spp. phytopathogens. To this end, we performed a detailed characterization of the impact of pEA29 and chromosomal thiamine biosynthesis genes in the production of EPS amylovoran in *E. amylovora*. We established

that a complete thiamine biosynthesis pathway was needed to produce amylovoran, the thiamine biosynthesis pathway positively regulates the function of the TCA cycle, and an actively functioning TCA cycle provides energetic requirements to produce amylovoran in *E. amylovora*.

RESULTS

Plasmid pEA29 is required for the virulence of *E. amylovora* in planta in a ThiOSGF-dependent manner.

Consistent with our previous studies (McGhee and Jones 2000; McGhee and Sundin 2008), the pEA29-cured strains of *E. amylovora* Ea110, hereafter named *E. amylovora* Ea110* in this study, showed reduced disease symptoms in immature pears relative to the wild-type (WT) parental strain Ea110 harboring the same empty vector pJB321 (Fig. 1A and B). At 6 days postinoculation, necrotic lesions and bacterial ooze were observed in Ea110/pJB321-inoculated pears (Fig. 1A) that were more severe than those inoculated with Ea110*/pJB321 showing decreased ooze production and reduced necrosis. In *trans* expression of the pEA29-derived thiamine biosynthesis operon *thiOSGF* (pJB321-*thiOSGF*) in Ea110* restored the virulence phenotype to WT levels (Fig. 1A and B). Interestingly, bacterial populations determined in pear tissue inoculated with WT or the plasmid-cured strains were not significantly different, suggesting that the reduced disease symptoms of Ea110* in immature pears is not due to a growth defect of Ea110* in planta (Supplementary Fig. S1). We also compared the bacterial growth of Ea110 and Ea110* in the Luria Bertani (LB) broth medium and minimal medium (MM), respectively, and no significant differences were observed (Supplementary Fig. S1).

To assess the impact of pEA29 on the virulence of *E. amylovora* in apple shoots, disease symptoms produced by Ea110/pJB321, Ea110*/pJB321, and the *thiOSGF*-complemented strains in apple shoots were measured 10 days after inoculation (Fig. 1B). Similar to the immature pear assay (Fig. 1A and B), a reduced lesion length was observed in shoots inoculated with Ea110*/pJB321 when compared with those inoculated with Ea110/pJB321 or complemented strains (Fig. 1C). The plasmid pJB321 had no impact on the disease progression of *E. amylovora* in both immature pear and apple shoot inoculation assays (data not shown). Together, our data support the conclusion that pEA29 is a bona fide virulence plasmid in *E. amylovora* and that the presence of the *thiOSGF* operon is required for full virulence.

Phylogenetic analysis of plasmid-derived thiamine biosynthesis genes in plant-associated bacteria.

A recent genomic analysis of a worldwide collection of 127 *E. amylovora* strains showed a nearly identical phylogeny produced using chromosomal or pEA29 sequences of *E. amylovora* (Parcey et al. 2020). To better understand the evolution of pEA29 and the role of the pEA29-derived *thiOSGF* operon in *E. amylovora*, we conducted a phylogenetic analysis using concatenated sequences of *thiO*, *thiS*, *thiG*, and *thiF* from pEA29 plasmids in 10 *E. amylovora* strains representing different geographic regions, host preference, and clades (Parcey et al. 2020; Zeng et al. 2018) and those on selected plasmids in other closely related Enterobacteriaceae species, such as *E. pyrifoliae* (92% identity to the pEA29-derived *thiOSGF*) and *Pantoea* spp. (76% identity to the pEA29-derived *thiOSGF*). Our data showed that the concatenated *thiOSGF* sequences of *E. amylovora* strains are phylogenetically located within the same clade and distant from those in the plant-pathogenic bacteria *E. pyrifoliae* DSM 12163 (Smits et al. 2010a), *E. piriflorinigrans* CFBP 5888 (López et al. 2011), and *Erwinia* sp. Ejp617 (Park et al. 2011) and more distant from those in the nonpathogenic bacteria *E. tasmaniensis* ET1/99 (Kube et al. 2008), *P. vagans* C9-1 (Smits et al. 2010c), and *P. agglomerans*

Fig. 1. Impact of pEA29 plasmid on *Erwinia amylovora* virulence and maximum-likelihood (ML) tree of *thiOSGF* genes. **A**, Disease symptoms in immature pears were examined in wild-type *E. amylovora* Ea110 harboring empty vector pJB321, Ea110* harboring pJB321, Ea110* harboring pJB321-*thiOSGF*, and phosphate-buffered saline (PBS) at 6 days postinoculation. **B**, Average lesion diameters of immature pears inoculated with the above-mentioned strains at 6 days postinoculation. **C**, Virulence of the above-mentioned strains was examined in apple shoots at 10 days postinoculation. The ratio of lesion length to shoot length for disease severity was calculated and assays were performed. All results are from one representative experiment. Two independent experiments were conducted, and three replicates (eight replicates for the immature pear assay) were used for each experiment. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means (not significant [ns] = $P > 0.05$ or $P < 0.05$ by Student's *t* test). **D**, ML tree based on concatenated sequences of genes *thiO*, *thiS*, *thiG*, and *thiF* from pEA29 plasmids of *E. amylovora*, selected plasmids of plant associated bacteria, including pPag3 of *Pantoea vagans* C9-1, pPagL15_1 of *P. agglomerans* L15, pJE01 of *Erwinia* sp. Ejp617, pEP36 of *E. pyrifoliae* DSM 12163, pEP37 of *E. piriflorinigrans* CFBP 5888, the chromosome of *E. tasmaniensis* ET1/99, and *Bacillus subtilis* subsp. *subtilis* strain 168. Sequences were retrieved from University of Wisconsin's ASAP and the National Center for Biotechnology Information GenBank databases. *E. amylovora* strains that belong to clades of Wide-Prevalent, B-group, Western North American, Eastern North American, and *Rubus*-infecting are labeled in purple, green, red, blue, and yellow, respectively (Parcey et al. 2020). Statistics of the ML model used to build the tree was GTR + G + I with 1,000 bootstraps. Nodes with bootstrap frequency 70% or greater are shown. *B. subtilis* was used as an outgroup. Scale bar indicates the number of substitutions per site of the genes.

L15 (Smits et al. 2015) (Fig. 1D). Similar to the phylogeny based on the entire sequences of the pEA29 plasmid (Parcey et al. 2020), phylogeny conducted using only the *thiOSGF* sequences displayed two clusters within *E. amylovora*, one cluster contains all eight *E. amylovora* strains in the *Amygdaloideae* (apple and pear)-infecting superclade and the other contains two *Rubus*-infecting superclade strains of *E. amylovora*, Ea644 and MR1 (Mann et al. 2013) (Fig. 1D). Thus, our data suggest that the pEA29-derived thiamine biosynthesis operon *thiOSGF* is highly conserved in *E. amylovora* and might function as a host-specificity factor involved in the regulation of the disease cycle.

Impact of pEA29 on T3SS gene expression, in vitro biofilm formation, and EPS production in *E. amylovora*.

The reduced disease symptoms of *E. amylovora* Ea110* in both immature pear assays and apple shoot inoculation assays (Fig. 1) encouraged us to investigate which pathogenicity or virulence factors of *E. amylovora* are affected by the pEA29 plasmid. Since the T3SS is one of the major pathogenicity factors (Oh and Beer 2005; Oh et al. 2005), we compared the promoter activities of three T3SS regulon genes, *hrpA* (encoding the T3SS pilus protein), *hrpN* (encoding a harpin protein), and *hrpL* (encoding the master regulator of T3SS), in Ea110* with those in WT (Fig. 2A). No significant difference was observed between WT and the plasmid-cured strains of *E. amylovora*, suggesting that the pEA29 plasmid is not involved in the regulation of T3SS gene expression in *E. amylovora*.

E. amylovora forms biofilms that have been characterized as a critical biological process in the disease cycle of fire blight (Koczan et al. 2009; Malnoy et al. 2012). We performed an in-vitro biofilm formation assay and found that Ea110*/pJB321 cells form approximately 40% less biofilm than does Ea110/pJB321 (Fig. 2B). Complementation in *trans* of the plasmid-derived *thiOSGF* operon in Ea110* restored the ability to produce the WT levels of biofilm (Fig. 2B), indicating that the pEA29-mediated effect on biofilm formation is dependent on the putative thiamine biosynthesis operon *thiOSGF* in *E. amylovora*. Since biofilm formation in *E. amylovora* relies on the production of three EPSs, namely, amylovoran, levan, and cellulose (Bellemann and Geider 1992; Castiblanco and Sundin 2018; Koczan et al. 2009), we further examined the impact of pEA29 on production of these EPSs. As shown in Figure 2C, the normalized OD₆₀₀ (optical density at 600 nm) value that represents the amount of secreted amylovoran in Ea110*/pJB321 was 2.4-fold lower than that in Ea110/pJB321. In line with our in-vitro biofilm formation assay (Fig. 2B), in *trans* complementation of *thiOSGF* increased the OD₆₀₀ values to WT levels in Ea110* (Fig. 2C). *E. amylovora* secretes the polymerizing enzyme levansucrase to produce levan from sucrose (Geider 2000; Seemüller and Beer 1977). Our data showed that the levansucrase activity of Ea110*/pJB321 is approximately 0.7-fold less than WT, whereas complementation of *thiOSGF* in *trans* significantly increased the activity of levansucrase in Ea110* to a level that is 3.8-fold higher than that in Ea110 harboring the empty vector (Fig. 2D). No difference was observed in terms of cellulose production when comparing Ea110* with Ea110 (Supplementary Fig. S2). Together, these data suggest that pEA29 is required for the formation of WT levels of biofilm in *E. amylovora*, and this mechanism is likely through modulating the production of amylovoran and levan.

in planta biofilm formation of *E. amylovora* WT and the plasmid-cured strains.

To assess the impact of pEA29 on the formation of biofilms inside the host vascular system, we used scanning electron microscopy (SEM) to examine cross sections of the petiole of apple leaves inoculated with WT Ea110, Ea110*, Ea110* containing pEA29 from *E. amylovora* BCN20 (a naturally occurring variant of pEA29 with the streptomycin-resistance transposon

Tn5393 inserted within *thiF*), and Ea110*/pEA29_{BCN20} complemented with *thiOSGF*. The WT strain *E. amylovora* Ea110 was localized in xylem vessels 7 days after infection (Fig. 3A and B). Cells that had attached to the inside of xylem vessels, formed multicellular aggregates, and expanded inward into the xylem vessel were observed in most cases. In addition, the production of a fibrillar material that links bacterial cells within the xylem vessels was observed for this strain (Fig. 3A and B). Different from Ea110, cells of Ea110* were not found in most xylem vessels (Fig. 3C and D). Fewer cell aggregates were observed with less complex structures for Ea110* than for Ea110 (Fig. 3C and D), suggesting that biofilm formation is greatly hindered in the pEA29-cured strain of *E. amylovora* in planta. In line with this conclusion, complementation of Ea110* with pEA29_{BCN20} also resulted in a strain that was deficient in biofilm formation within xylem vessels (Fig. 3E and F). Expression of the *thiOSGF* operon in *trans* in Ea110*/pEA29_{BCN20} restored the multicellular aggregate phenotype and mature biofilm formation in the host vascular tissues (Fig. 3G and H).

Compositional analysis of EPS from WT and the plasmid-cured strains of *E. amylovora*.

Studies from different *E. amylovora* strains, including Ea110, have reported altered colony morphology when the plasmid-cured strains of *E. amylovora* were growing on MM lacking thiamine (MM-T), indicating a defect in the production of EPS in these strains (Falkenstein et al. 1989; McGhee and Jones 2000). We visualized the bacterial growth of Ea110 and Ea110* on MM-T in vitro and found that Ea110 cells were large and turbid with a mucoid morphology, whereas Ea110* cells were small and translucent with a mucoid morphology. In *trans* complementation of *thiOSGF* in Ea110* restored this phenotype and also resulted in an increased production of EPS compared with that in Ea110 harboring the empty vector, likely due to the copy effect of the complementation plasmid (Supplementary Fig. S3).

We next analyzed the EPS produced by the *E. amylovora* strains that exhibited drastic morphological differences following growth on MM-T. WT Ea110, Ea110*, and Ea110*/pEA29_{BCN20} were grown in MM-T broth medium for 48 h (Ea110) or 96 h (Ea110* and Ea110*/pEA29_{BCN20}), following which the EPS was precipitated, was dialyzed with deionized water, and was lyophilized. Lyophilized EPS was subject to gas chromatography-mass spectrometry (GC-MS) analysis, and the results indicated that the EPS produced by *E. amylovora* Ea110 consisted of sugar components of amylovoran, including galactose, glucuronic acid, and glucose (Table 1). In contrast, the EPS produced by *E. amylovora* Ea110* and Ea110*/pEA29_{BCN20} following growth in MM-T did not contain any detectable glucuronic acid, as compared with WT Ea110 (Table 1). The amylovoran structure consists of a repeating subunit that contains galactose and glucuronic acid in an approximate 4:1 ratio with about 1,000 subunits comprising an individual amylovoran molecule (Nimtz et al. 1996). An additional glucose residue is linked to ~10% of the core α -galactose of the repeating unit (Nimtz et al. 1996). Our results for the Ea110 EPS were in line with this, suggesting that we were identifying amylovoran produced from this strain and that amylovoran biosynthesis is compromised in Ea110* and in Ea110* complemented with a pEA29 plasmid derivative with an insertional mutation in *thiF*.

To further validate this conclusion, we deleted the chromosomally encoded *ugd* gene, encoding a putative UDP-glucose dehydrogenase involved in the production of glucuronic acid, in *E. amylovora* Ea1189 and examined the effect of this mutation on amylovoran production and bacterial growth on MM-T. We used strain Ea1189 for these experiments because this strain is more amenable to genetic manipulation than strain Ea110. Deletion of *ugd* significantly decreased the production of amylovoran by 3.4-fold in comparison with the WT Ea1189 harboring the same empty vector (Fig. 4B), and Ea1189 Δ *ugd* cells produced a translucent and mucoid morphology

similar to the pEA29-cured strains (Supplementary Fig. S3). Complementation of Ea1189 Δ ugd in *trans* restored the mutant phenotype to WT levels (Fig. 4B; Supplementary Fig. 3).

The pEA29 plasmid has no impact on the transcription or messenger RNA (mRNA) levels of *ams* genes.

To further understand the molecular mechanism for the pEA29-mediated effect on amylovoran biosynthesis, we first examined the

promoter activity of *amsG*, the first gene in the *ams* operon, in Ea110 and Ea110*. As shown in Figure 4C, the promoter activity of *amsG* was not altered in Ea110* when compared with the WT Ea110, suggesting that the defective production of amylovoran in Ea110* is not through the transcriptional regulation of *ams* genes. Consistent with the transcriptional data (Fig. 4C), mRNA levels of two *ams* genes, *amsG* and *amsF*, were not reduced in Ea110* compared with those in Ea110 (Fig. 4D). In contrast, mRNA

Fig. 2. pEA29 plays important roles in in-vitro biofilm formation and exopolysaccharide production in *Erwinia amylovora*. **A**, Transcriptional activities of *hrpL*, *hrpA*, and *hrpN* were measured in wild-type (WT) *E. amylovora* Ea110 and Ea110* strains and relative fluorescence intensity was calculated. **B**, In vitro biofilm formation, **C**, amylovoran production, and **D**, levansucrase activities were determined in WT *E. amylovora* Ea110 harboring the empty vector pJB321, Ea110* harboring pJB321, and Ea110* harboring pJB321-*thiOSGF*. Assays were performed, one representative experiment was chosen, and three independent experiments were performed. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means (one [*] indicates $P < 0.05$ and two [**] $P < 0.01$, by Student's *t* test). ns = not significant.

levels of two plasmid-derived thiamine biosynthesis genes, *thiO* and *thiG*, were significantly reduced in the plasmid-cured strains of *E. amylovora* (Fig. 4D).

Addition of thiamine positively regulates amylovoran production in WT and plasmid-cured strains of *E. amylovora*.

If the plasmid pEA29 positively regulates amylovoran production in *E. amylovora* indeed via plasmid-derived thiamine biosynthesis genes *thiO*, *thiS*, *thiG*, and *thiF*, we surmised that addition of exogenous thiamine could restore the production of amylovoran in *E. amylovora* Ea110*. As expected, addition of 0.1% (wt/vol) of exogenous thiamine increased the production of amylovoran in Ea110* to WT levels (Fig. 5A), suggesting that the reduced production of amylovoran in Ea110* is due to its inability to produce thiamine. Furthermore, addition of 0.4, 0.8, and 1.6% of exogenous thiamine increased the production of amylovoran by 1.3-, 1.9-, and 2.7-fold, respectively, when compared with that in the presence of 0.1% of thiamine in Ea110* (Fig. 5A). Similar induction rates of the production of amylovoran were also observed in Ea110 in the presence of thiamine (Fig. 5A), suggesting that thiamine induces the production of amylovoran in a dose-dependent manner in *E. amylovora*. To assess the impact of bacterial growth on the thiamine-mediated induction of amylovoran in *E. amylovora*, we measured the growth of Ea110 in the absence or presence of exogenous thiamine (0.4 and 0.8%, respectively) and calculated the amylovoran production based on cell number. Similar to the amylovoran production calculated based on the OD₆₀₀ values, addition of 0.4 or 0.8% of thiamine significantly increased the amylovoran production in *E. amylovora* relative to that in the absence of thiamine (Supplementary Fig. S4).

Next, we questioned whether the thiamine-dependent induction of amylovoran occurs in other *E. amylovora* strains, and so the production of amylovoran was measured in WT *E. amylovora* Ea1189 and Ea1189Δ*ams*, a mutant that lacks the chromosomal amylovoran biosynthesis genes, in the presence or absence of exogenous thiamine. Despite a lower level of amylovoran detected in WT Ea1189 relative to Ea110 in the absence of exogenous thiamine, addition of thiamine increased the production of amylovoran in Ea1189 in a dose-dependent manner similar to that in Ea110 (Fig. 5B). On the other hand, the *ams* mutant failed to produce amylovoran regardless of thiamine addition, indicating that the thiamine-mediated amylovoran production requires the

presence of amylovoran biosynthesis genes. However, consistent with the result from the plasmid-cured strains of *E. amylovora* (Fig. 4C), addition of exogenous thiamine had no impact on the transcription of *ams* genes in both Ea110 and Ea1189 (Fig. 5C).

Products of chromosomal thiamine biosynthesis genes are required to produce amylovoran.

The thiamine biosynthesis pathway has been characterized in bacteria such as *Escherichia coli* and *Bacillus subtilis* (Jurgenson et al. 2009; Rodionov et al. 2002). We performed a BLAST search of *E. amylovora* using query sequences of amino acid sequences of selected *Escherichia coli* thiamine biosynthetic enzymes and identified homologs (>96% coverage and >70% identity) that are chromosomally encoded in *E. amylovora*. We then examined the effect of these chromosomal thiamine biosynthesis genes on amylovoran production. As shown in Figure 6, deletion of the *thiL* gene (encoding product involved in hydroxyethylthiazole monophosphate [HET-P] synthesis [Webb et al. 1997]) or the *thiCE* operon (ThiC and ThiE are involved in hydroxymethylpyrimidine pyrophosphate [HMP-PP] synthesis and thiamine monophosphate synthesis via coupling HET-P and HMP-PP, respectively [Backstrom et al. 1995; Vander Horn et al. 1993]) in *E. amylovora* Ea1189 significantly reduced the production of amylovoran by 2.4- and twofold relative to the WT Ea1189, respectively (Fig. 6A). A complementation assay restored amylovoran production in the mutants to WT levels (Fig. 6A). Deletion of the *thiMD* operon (ThiM and ThiD are involved in salvage of HET and HMP from the surrounding environment, respectively [Petersen and Downs 1997]) only slightly reduced the production of amylovoran (Fig. 6A), whereas overexpression of *thiMD* in *trans* in Ea1189 increased amylovoran production by approximately 1.5-fold compared with

Table 1. Compositional analysis of exopolysaccharide (EPS) from *Erwinia amylovora* Ea110, Ea110*, and BCN20

EPS	Mole %		
	Ea110	Ea110*	Ea110*/pEA29 _{BCN20}
Fucose	2.4	8.9	—
Xylose	0.9	3.6	—
Glucuronic acid	15.1	—	—
Galactose	66.4	29.9	35.6
Glucose	15.2	55.6	54.6
Mannose	—	1.9	9.8

Fig. 3. Visualization of biofilm formation by **A** and **B**, wild-type *Erwinia amylovora* Ea110, **C** and **D**, Ea110*, **E** and **F**, Ea110* harboring plasmid pEA29_{BCN20}, and **G** and **H**, Ea110* harboring pEA29_{BCN20} and pJB321-*thiOSGF* strains in apple leaves using scanning electron microscopy. Bars = 10 μm in A, C, E, and G and 1 μm in B, D, F, and H.

the same strain harboring an empty vector (Fig. 6A). Functions of the chromosomal thiamine synthesis genes were evaluated in an apple shoot infection assay, showing that the deletion of either *thiCE* or *thiL* significantly reduced the virulence of *E. amylovora* in planta (Fig. 6B). The product of *thiL* is thought to catalyze the phosphorylation of thiamine monophosphate to TPP (Webb and Downs 1997). Despite several attempts, deletion of *thiL* were not successful. Taken together, our data suggest that a complete thiamine biosynthesis pathway consisting of plasmid and chromosomal thiamine biosynthesis genes or operons is necessary for the

full-level production of amylovoran and in planta virulence in *E. amylovora*.

Impact of bacterial respiration on amylovoran production in *E. amylovora*.

The thiamine biosynthesis pathway produces TPP, which is an essential cofactor of several enzymes of metabolic importance such as pyruvate dehydrogenase and α -ketoglutarate dehydrogenase (Dunn 1998; Sudarsan et al. 2005). To investigate the function of TPP-dependent enzymes in amylovoran production,

Fig. 4. Impacts of pEA29 on bacterial morphology and amylovoran production in *Erwinia amylovora*. **A**, Bacterial morphology of wild-type (WT) *E. amylovora* Ea110 and Ea110* on minimal medium lacking thiamine was determined at 4 days postinoculation. Microphotographs were captured using a light microscopy with incident lighting (upper graphs) or transmitted lighting (lower graphs). The scale bar represents 1.0 cm. **B**, Amylovoran production was determined in WT *E. amylovora* Ea1189 harboring the empty vector pBBR1-MCS5, Δ ugd harboring pBBR1-MCS5, and Δ ugd harboring pBBR1-ugd. **C**, The expression levels of *amsG* were determined in Ea110 and Ea110* strains and relative fluorescence intensity was calculated. **D**, RNA levels of *amsG*, *amsF*, *thiO*, and *thiG* were measured in Ea110 and Ea110* strains. One representative experiment was chosen, and three independent experiments with three replicates were performed. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ($P < 0.05$ by Student's *t* test). ns = not significant.

we constructed two mutants, Ea1189 $\Delta aceE$ (pyruvate dehydrogenase E1 component–encoding gene) and Ea1189 $\Delta sucA$ (α -ketoglutarate dehydrogenase E1 component–encoding gene) and examined the production of amylovoran in these mutant strains. Our results showed that both mutant strains are unable to produce amylovoran and are immune to the addition of exogenous thiamine, similar to the *ams* mutant (Fig. 7A). In *trans* expression

of *sucA* restored the mutant phenotype to WT levels and in *trans* expression of *aceE* in Ea1189 $\Delta aceE$ increased the amylovoran production to a level that was sixfold higher than in WT Ea1189 (Supplementary Fig. S5).

Since *SucA* and *AceE* are key enzymes involved in the TCA cycle, we hypothesized that a functional TCA cycle is required for *E. amylovora* to produce amylovoran. To test this, we measured

Fig. 5. Impacts of exogenous thiamine on amylovoran production in *Erwinia amylovora*. **A**, Amylovoran production was measured in wild-type (WT) *E. amylovora* Ea110 and Ea110* strains with or without the supplementation of 0.1, 0.2, 0.4, 0.8, or 1.6% (wt/vol) of thiamine, and in **B**, WT *E. amylovora* Ea1189 and Δams with or without the supplementation of 0.4, 0.8, or 1.6% of thiamine. **C**, The promoter activity of *amsG* was measured in Ea110 and Ea1189 in the absence or presence of 0.4% of thiamine and relative fluorescence intensity was calculated. Three independent experiments were performed with three replicates in each experiment. Values are from one representative experiment. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ($P < 0.05$ by Student's *t* test). ns = not significant.

Fig. 6. Chromosomal thiamine biosynthesis genes are required for amylovoran production and virulence of *Erwinia amylovora*. **A**, Amylovoran production was measured in WT *E. amylovora* Ea1189 harboring empty vector pBBR1-MCS5, $\Delta thiCE$ harboring pBBR1-MCS5, $\Delta thiCE$ harboring pBBR1-*thiCE*, $\Delta thiI$ harboring pBBR1-MCS5, $\Delta thiI$ harboring pBBR1-*thiI*, $\Delta thiMD$ harboring pBBR1-MCS5, and Ea1189 harboring pBBR1-*thiMD* strains. Values are representative of three independent experiments. Three replicates were used in each experiment. Error bars indicate standard errors of the means. Different lowercase letters above the bars indicate statistically significant differences between treatments (Fisher's least significant difference, $P < 0.05$). **B**, Virulence of Ea1189, $\Delta thiCE$, and $\Delta thiI$ strains was examined in apple shoots at 10 days postinoculation. The ratio of lesion/shoot length for disease severity was calculated. Two independent experiments with at least three biological replicates were performed. Data are from one representative experiment. Error bars indicate standard errors of the means. Asterisks indicate statistically significant differences of the means ($P < 0.05$ by Student's *t* test).

the production of amylovoran in *E. amylovora* cells grown in a semi-anaerobic condition in which TCA cycle-dependent aerobic respiration is not favored. We first compared the bacterial growth of *E. amylovora* under aerobic and semi-anaerobic conditions. Our data showed that OD₆₀₀ values of *E. amylovora* Ea110 cells cultured under semi-anaerobic conditions are significantly lower than those grown in aerobic conditions at both the exponential and stationary growth phases (Supplementary Fig. S6). However, similar growth curves were observed for both culture conditions allowing us to conduct the following experiments at 24 h postinoculation, equivalent to the early stationary growth phase (Supplementary Fig. S6). As we hypothesized, the production of amylovoran by *E. amylovora* was reduced by 2.3-fold when the TCA cycle was impaired (Fig. 7B). More importantly, addition of exogenous thiamine or in *trans* expression of *thiOSGF* restored the production of amylovoran in *E. amylovora* Ea110 to aerobic-condition levels when cells were grown in the semi-anaerobic condition and had no impact on bacterial growth (Fig. 7B; Supplementary Fig. 6), implying a regulatory signaling between the TCA cycle and thiamine biosynthesis in the regulation of amylovoran in *E. amylovora*. In addition, no significant differences in the expression of *ams* or *thi* genes were observed in *E. amylovora* Ea110 between the normal aerobic and semi-anaerobic

growth conditions (Fig. 7C and D). These data agree with the notion that the thiamine-mediated effect on amylovoran biosynthesis is independent of the regulation of *ams* gene expression.

DISCUSSION

In this study, we determined that the presence of the ubiquitous plasmid pEA29 was required for full virulence and proper localization of *E. amylovora* cells in xylem vessels during plant infection and that the *thiOSGF* operon was the critical virulence factor encoded on this plasmid. Thiamine biosynthesis by *E. amylovora* was necessary for the cells to produce amylovoran because, as our data showed, the EPS produced by cells lacking the *thiOSGF* operon or with an insertional mutation in *thiF* did not contain the key glucuronic acid component of amylovoran. Interestingly, the expression of *ams* genes was not reduced in the pEA29-cured strain of *E. amylovora* relative to the WT parental strain, suggesting that the thiamine-mediated effect on amylovoran production is indirect. We further validated this hypothesis by examining the impact of exogenous thiamine on amylovoran production in *E. amylovora* and found that addition of exogenous thiamine restored amylovoran production in the pEA29-cured strain without affecting the expression of *ams* genes. We also

Fig. 7. The tricarboxylic acid cycle positively contributes to amylovoran production in *Erwinia amylovora*. **A**, Amylovoran production was measured in wild-type (WT) *E. amylovora* Ea1189, Δ*sucA*, Δ*aceE*, and Δ*ams* strains in the absence or presence of exogenous thiamine, and in **B**, WT *E. amylovora* Ea110, Ea110 harboring the empty vector pJB321, and Ea110 harboring pJB321-*thiOSGF* strains under aerobic (O₂⁺) or semi-anaerobic (O₂⁻) conditions with or without the supplementation of thiamine. **C**, Transcriptional activity of *amsG* gene and **D**, RNA levels of *amsG*, *amsF*, *thiO*, and *thiG* were measured in WT *E. amylovora* Ea110 under aerobic and semi-anaerobic conditions, respectively. The O₂⁺/O₂⁻ ratio for each gene expression and relative fluorescence intensity were calculated. Values are representative of three independent experiments. Three replicates were used in each experiment. Error bars indicate standard errors of the means. ns = not significant. Asterisks indicate statistically significant differences of the means (*P* < 0.05 by Student's *t* test).

showed that a complete thiamine biosynthesis pathway, consisting of enzymes encoded by the *thiOSGF* operon of pEA29 and other chromosomally encoded thiamine biosynthesis genes, was required for amylovoran production. The TCA cycle functions in respiration metabolism and plays an essential role in bacterial growth under aerobic conditions. We showed that a functional TCA cycle positively regulates amylovoran production in *E. amylovora*. It is likely that TPP, an active form of thiamine, serves as a cofactor of enzymes of glycolysis and the TCA cycle, which, in turn, provides high energy molecules such as adenosine triphosphate for the metabolism of precursors of amylovoran in *E. amylovora* (Fig. 8).

The thiamine biosynthesis pathway has been solved for bacteria including *Escherichia coli*, and the key components utilized in synthesis include glyceraldehyde 3-phosphate, pyruvate, tyrosine or glycine, and a sulfur atom from the ThiS protein (Jurgenson et al. 2009). At least 18 genes are involved in the core reactions resulting in thiamine synthesis (Rodionov et al. 2002). In *Escherichia coli*, the ThiH protein functions in the conversion of tyrosine to dehydroglycine; in contrast, *B. subtilis* utilizes the ThiO protein to convert glycine to dehydroglycine in this initial step in thiamine synthesis (Jurgenson et al. 2009). Although *E. amylovora*, like *Escherichia coli*, is a member of the Enterobacteriaceae family, the presence of the *thiO* gene and absence of *thiH* in the genome (data not shown) suggests that glycine is the amino acid converted to dehydroglycine in *E. amylovora* during the initial steps of thiamine synthesis.

In *Escherichia coli*, the thiamine biosynthetic genes are clustered within three chromosomal operons, *thiCEFSGH*, *thiMD*,

and *thiBPQ* (which encodes for a thiamine transport system), and there are four additional single genes, *thiL*, *dxs*, *thiL*, and *thiK* (Begley et al. 1999). The operons each contain a highly conserved *thi* box sequence in the 5' untranslated region of the mRNA and are regulated by a riboswitch mechanism that is responsive to TPP (Miranda-Ríos et al. 2001). The single genes are not regulated, and some of these genes (*dxs*, *thiL*) are involved in other metabolic pathways. In *E. amylovora*, the genetic organization of *thiMD* and *thiBPQ* in operons is conserved, single genes *dxs*, *thiL*, and *thiK* are present, and the *thiL* gene is present and adjacent to an additional gene, *thiJ*. *thiC* and *thiE* are present as an operon but lack the additional four genes (*thiF*, *thiS*, *thiG*, and *thiH*) present in *Escherichia coli*. Instead, a similar set of genes is present on pEA29 as *thiOSGF*. We analyzed the sequences upstream of *thiMD* and *thiBPQ* visually in *E. amylovora* ATCC 49946 and identified *thi* box sequences that were closely related (72 to 90% nucleotide identity) to the *thi* box upstream of *thiC* in *Escherichia coli* and *Salmonella typhimurium* (Miranda-Ríos et al. 1997). However, a *thi* box was not detected upstream of the pEA29-encoded *thiOSGF* operon.

The *thiOSGF* operon structure is not unique to *E. amylovora*, as similar operons are present on plasmids in related pathogens, such as *E. pyrifoliae* (Maxson-Stein et al. 2003) and *E. piriflorinigra* (López et al. 2011), on plasmids from nonpathogenic *Pantoea* spp. strains such as *P. vagans* (De Maayer et al. 2012), and on the chromosome of the nonpathogenic *E. tasmaniensis* (Kube et al. 2010). In addition, draft sequences of a group of 20 large plasmids from seven *P. species*, ranging from 281 to 794 kb and collectively termed the LPP-1 (large *Pantoea* plasmid) group, revealed that all

Fig. 8. Working model for the thiamine biosynthesis pathway-mediated regulation of amylovoran production in *Erwinia amylovora*. Enzymes encoded by the pEA29 plasmid-derived *thiOSGF* operon and selected chromosomal *thi* genes, including *thiC*, *thiE*, *thiL*, *thiM*, and *thiD*, are essential for the process of thiamine biosynthesis in *E. amylovora*. Thiamine pyrophosphate (TPP) synthesized by the thiamine biosynthesis pathway functions as a cofactor that interacts with pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, resulting in positive contribution to glycolysis and the tricarboxylic acid (TCA) cycle, respectively. Glucose 6-phosphate (glu-6-P) synthesized from glucose or fructose and sorbitol serve as a major source for uridine diphosphate-(UDP-) sugar metabolism, which also requires two high-energy molecules, uridine triphosphate (UTP), and the TCA cycle-produced adenosine triphosphate. Sugars including UDP-glucose (UDP-Glc), UDP-galactose (UDP-Gal), and UDP-glucuronic acid (UDP-GlcA) are main precursors for the biosynthesis of amylovoran in *E. amylovora*. The dotted lines indicate regulatory mechanisms identified in this study.

of these plasmids shared the *thiOSGF* operon (De Maayer et al. 2012). Also, *thiOSGF* is encoded on the 530-kb megaplasmid pPag3 from the biocontrol strain *P. vagans* C9-1 (Smits et al. 2010c). A variant of *P. vagans* C9-1 that had lost pPag3 only grew with thiamine supplementation on MM amended with glucose, suggesting that the plasmid-encoded *thiO*, *thiS*, *thiG*, and *thiF* genes were required for thiamine biosynthesis in this organism (Smits et al. 2010c). Our phylogenetic analysis using concatenated sequences of *thiOSGF* revealed that the *thiOSGF* operon is highly conserved in *E. amylovora* and is closely related to those on plasmids in other pathogenic *Erwinia* spp. but is distant from the chromosomal *thiOSGF* operon of *E. tasmaniensis* and non-pathogenic *Pantoea* spp. Nevertheless, it remains to be determined why the *thiOSGF* operon for thiamine biosynthesis is encoded in a plasmid rather than the chromosome of *E. amylovora* as well as if this arrangement plays a role in modulating interactions of *E. amylovora* with hosts.

Several studies have shown that thiamine, most likely through downstream functions of TPP-dependent enzymes, affects bacterial phenotypes such as host colonization and virulence. For example, a *Listeria monocytogenes thiT* mutant deficient in thiamine uptake displayed high acid sensitivity, presumably due to the thiamine dependence of the enzyme acetolactate synthase (Madeo et al. 2012). Acid tolerance is a critical requirement for survival of *L. monocytogenes* during colonization of the host gastrointestinal tract. A *thiC* mutant of *Pseudomonas fluorescens* CHA0 produced about 25-fold less signal molecule active in cell-to-cell signaling when grown in the presence of a very low (5×10^{-8} M) level of exogenous thiamine (Dubuis et al. 2006). Similarly, a thiamine auxotroph of *Pseudomonas fluorescens* WCS365 was unable to colonize tomato roots under axenic conditions (Simons et al. 1996). In the rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae*, virulence was reduced in a *thiG* mutant as the mutant exhibited reduced motility and migration in planta in rice leaves (Yu et al. 2015). The authors demonstrated that the expression of the regulatory genes *rpfC* and *rpfG* was reduced in the *thiG* mutant leading to a decrease in cell-to-cell aggregation, which was important for biofilm formation and virulence (Yu et al. 2015). Intriguingly, thiamine was also potentially linked to cyclic di-GMP regulation in *X. oryzae* pv. *oryzae*; we have shown that this second messenger molecule also plays an important role in regulating all aspects of virulence in *E. amylovora* (Edmunds et al. 2013; Kharadi et al. 2019).

From our results, the mechanistic role of thiamine in the virulence of *E. amylovora* appears to be through an impact on the production of amylovoran EPS. Amylovoran is a pathogenicity factor, as amylovoran-deficient mutants are completely non-pathogenic in both immature pear and apple tree infection models (Geider 2000; Zhao et al. 2009). Amylovoran has been hypothesized to protect cells from host surveillance and disease resistance responses (Geider 2000). Yet, amylovoran is also a key component of the biofilm developmental phase, which is critical for *E. amylovora* virulence and is expressed later in the infection cycle when cells invade plant xylem vessels (Koczan et al. 2009, 2011). The biosynthesis and polymerization of amylovoran requires the *ams* amylovoran-biosynthetic operon (Bugert and Geider 1995). It is believed that assembly of the repeating unit of amylovoran is initiated by AmsG via attaching the first galactose residue to the lipid carrier, undecaprenyl-phosphate. *amsG* mutants of *E. amylovora* were unable to incorporate ^{14}C -UDP-galactose (Langlotz et al. 1999). Subsequent sugars are attached sequentially and are dependent on AmsD, AmsC, AmsB, AmsK, AmsE, and AmsJ (Geider 2000). Translocation and polymerization of the repeating units have been reported to be associated with AmsH, AmsA, AmsF, and AmsL (Bugert and Geider 1997; Ilan et al. 1999). UDP sugar metabolism is clearly essential for the synthesis of amylovoran in *E. amylovora*, as our data showed that deletion of

the *ugd* gene, encoding the putative UDP-glucose dehydrogenase that converts UDP-glucose to UDP-glucuronic acid, greatly attenuated amylovoran production. At the level of gene regulation, the *ams* operon is regulated in response to environmental conditions such as carbon source availability, temperature, and pH (Wang et al. 2011b). Expression of the *amsG* promoter is positively controlled by the Rcs phosphorelay system, other regulatory genes, such as *amyR*, and is also controlled by factors such as elevated cellular cyclic di-GMP level (Edmunds et al. 2013; Wang et al. 2012a and b). However, we found that the requirement of thiamine in amylovoran production is indirect and related to the role of thiamine as a cofactor in enzymes affecting TCA cycle function, as an actively functioning TCA cycle is essential for the energetic requirements of amylovoran synthesis, polymerization, and secretion.

A wide range of bacterial species use the TCA cycle to control cellular behaviors and virulence factors (Bücker et al. 2014; Ding et al. 2014; Takeuchi et al. 2009). A *Pseudomonas aeruginosa aceAB* mutant exhibits a greatly attenuated T3SS and is non-pathogenic in a rat model (Dacheux et al. 2002). AceA and AceB are major components of pyruvate dehydrogenase, which converts pyruvate to acetyl-CoA (Rae et al. 1997). In the soft rot plant pathogen *Dickeya dadantii*, deletions of two key TCA cycle enzyme-encoding genes, *fumA* (encoding fumarase) and *sdhCDAB* (encoding succinate dehydrogenase), resulted in reduced intracellular levels of cyclic di-GMP and increased production of a plant cell wall-degrading enzyme pectate lyase (Yuan et al. 2020). Since *D. dadantii* encounters aerobic and anaerobic conditions during spread and infection and low oxygen availability in the plant apoplast has been shown to stimulate the production of pectate lyase (Hugouvieux-Cotte-Pattat et al. 1992; Reverchon and Nasser 2013), we proposed that the TCA cycle and TCA cycle metabolites could serve as signals for the regulation of virulence in bacteria (Yuan et al. 2020). In the *Erwinia*-apple pathosystem, connections between bacterial respiration, fitness, and virulence are poorly understood. Puławska et al. (2017) conducted a transcriptome analysis of a low-virulence strain of *E. amylovora* during shoot infections of susceptible ('Idared') and resistant ('Free Redstar') apple cultivars, showing that the thiamine biosynthesis genes *thiO*, *thiS*, *thiG*, and *thiF*, located on the pEA29 plasmid were up-regulated in planta as well as genes involved in amylovoran biosynthesis and the T3SS at 24 h postinoculation. In addition, almost all genes involved in the TCA cycle, including *glTA* (citrate synthase-encoding gene), *aceE* and *aceF* (pyruvate dehydrogenase-encoding genes), *sucA* and *sucB* (α -ketoglutarate dehydrogenase-encoding genes), *sucC* and *sucD* (succinyl-CoA synthetase-encoding genes), *sdhC*, *sdhD*, *sdhA*, and *sdhB*, and *fumC* (fumarase-encoding gene), were down-regulated in planta, whereas the chromosomally located thiamine biosynthesis genes *thiCE* and *thiL* were up-regulated (Puławska et al. 2017). A recent study identified a series of auxotrophic *E. amylovora* mutants that exhibit reduced virulence in immature apple (*Malus pumila*) fruits compared with the WT strain (Klee et al. 2019). These mutants include those defective in glycolysis, which is in line with our findings and those involved in purine and pyrimidine metabolism that contribute to thiamine biosynthesis (Rodionov et al. 2002). We showed that a complete thiamine biosynthesis pathway is required for amylovoran production and virulence of *E. amylovora*, as cells lacking chromosomal or plasmid *thi* genes were unable to produce biofilms in xylem vessels and exhibited reduced overall virulence symptoms in planta. Together, these data suggest that it is likely that *E. amylovora* encounters a low-oxygen environment during an infection and the ability to produce thiamine is needed for amylovoran production and biofilm formation under this condition.

pEA29 is present in almost all *E. amylovora* isolates recovered worldwide; in fact, for many years, the main detection protocol for *E. amylovora* utilized PCR primers that matched a sequence on the plasmid (Bereswill et al. 1992). However, in recent years, a small

number of *E. amylovora* strains have been isolated that lack this plasmid (Llop et al. 2006; Mohammadi et al. 2009). Some *E. amylovora* strains lacking pEA29 that were isolated in Spain and Europe contained a novel plasmid, pEI70, that contributed to virulence, as these strains retained high virulence to apple and pear (Llop et al. 2011). However, other natural *E. amylovora* strains lacking pEA29 were reduced in virulence, as addition of pEA29 to plasmid-free natural *E. amylovora* strains isolated in Egypt resulted in increased amylovoran production and increased tissue necrosis and bacterial oozing in an immature pear infection model (Mohammadi 2010). Although the sharply-reduced virulence of some natural strains lacking pEA29 seems contradictory for a plant pathogen, there are a few possible explanations for the survival of these strains: i) strains lacking pEA29 could be complemented in Rosaceae family plant hosts through the utilization of thiamine produced by the host; and ii) it has been shown in laboratory studies that two pathogenicity mutants of *E. amylovora* can be complemented in planta through mixing, as long as all traits necessary for pathogenicity are present among the two mixed strains (Koczan et al. 2009; Zhao et al. 2009). Thus, strains lacking pEA29 with low virulence may hitchhike in planta with other fully virulent strains and remain viable in mixed-strain infections. This explanation could also extend to the low-virulence strain *E. amylovora* BCN20 from this study, which is a natural isolate from Michigan with a Tn5393 insertion in the *thiF* gene.

Overall, our results showed that the involvement of pEA29 in *E. amylovora* virulence is mainly through the presence of the *thiOSGF* operon that functions in thiamine biosynthesis and is required for amylovoran production in the absence of exogenous thiamine. TPP stimulates the function of the TCA cycle, providing energy requirements for the biosynthesis of amylovoran. Thus, pEA29 appears to be ubiquitous in *E. amylovora* because of its importance to virulence, as *E. amylovora* strains do not grow and develop large populations in nature in the absence of host infection.

MATERIALS AND METHODS

Strains, plasmids, primers, and media.

The bacterial strains and plasmids used in this study are listed in Table 2. *E. amylovora* and *Escherichia coli* strains were grown in LB broth medium at 28 and 37°C, respectively. Amylovoran production assays were conducted in modified basal medium A (MBMA) (Edmunds et al. 2013) amended with 1% (wt/vol) of sorbitol. In vitro biofilm formation assays were conducted in 0.5× LB medium. The expression of T3SS genes was measured in cells cultured in hypersensitive response and pathogenicity (Hrp)-inducing MM to induce the expression of the T3SS regulon (Huynh et al. 1989). Carbohydrate analysis experiments were conducted in cells cultured in MM-T (Na₂HPO₄ at 6 g/liter, KH₂PO₄ at 3 g/liter, NaCl at 0.5 g/liter, NH₄Cl at 1 g/liter, MgSO₄·7H₂O at 0.2 g/liter, CaCl₂ at 0.1 g/liter, nicotinic acid at 0.2 g/liter, and sucrose at 20 g/liter (Falkenstein et al. 1989)). Antibiotics were added to the media as follows: ampicillin (100 µg/ml), chloramphenicol (10 µg/ml), gentamicin (15 µg/ml), or kanamycin (30 µg/ml). The *E. amylovora* genome sequence was retrieved from a systematic annotation package for community analysis of genomes (ASAP database). Primers used for cloning are listed in Supplementary Table S1.

Virulence assay.

The virulence of *E. amylovora* WT Ea110, Ea110*, and the *thiOSGF*-complemented strains was tested using an immature pear fruit (*Pyrus communis* cv. Bartlett) assay and an apple shoot (*Malus × domestica* cv. Gala) assay as previously described (Wang et al. 2011a; Zhao et al. 2005). Briefly, for the immature pear assay, 2 µl of bacterial suspension in phosphate-buffered saline (PBS) at 10⁴ CFU/ml were inoculated into the stab-wounded immature pears. Inoculated immature pears were incubated at 28°C

under high-humidity conditions. To evaluate disease symptoms, pears were photographed and were assessed for water soaking or necrotic symptoms at 4, 5, and 6 days postinoculation. Lesion diameters were measured at 6 days postinoculation. Bacterial populations within immature pear fruits were quantified at 1, 2, 3, and 4 days postinoculation. For the apple shoot assay, the youngest apple leaves were inoculated by cutting with scissors dipped in bacterial suspension of 2 × 10⁸ CFU/ml. The length of the necrotic lesion was measured regularly from the point of inoculation. All assays were repeated three times, with a minimum of three technical replicates per treatment in each experiment.

Bioinformatics and phylogenetics analysis.

The nucleic acid sequences of thiamine biosynthesis genes were aligned using T-Coffee (Notredame et al. 2000) and were edited manually. Sequences of thiamine biosynthesis genes were retrieved from ASAP and the National Center for Biotechnology Information GenBank databases. *E. amylovora* CFBP 1430 (accession GCA_000091565.1), *E. amylovora* CFBP 2585 (CA OZ000000000), *E. amylovora* ATCC 49946 (GCA_000027205), *E. amylovora* Ea110 (NQJZ000000000), *E. amylovora* Fb-97b (JAAEUP000000000), *E. amylovora* LA472 (JAAEUM000000000), *E. amylovora* CTBT1-1 (NQJP000000000), *E. amylovora* ATCC BAA-2158 (PRJEA52823), *E. amylovora* MR1 (CAPE000000000), *E. amylovora* Ea644 (CAPD000000000), *E. piriflorinigrans* CFBP 5888 (CAHS010000000), *Erwinia* sp. Ejp617 (NC_017445), *E. pyrifoliae* DSM 12163 (FN392235), *E. tasmaniensis* ET1/99 (NC_010694), *P. vagans* C9-1 (CP002206), *P. agglomerans* L15 (CP034148), and *Bacillus subtilis* subsp. *subtilis* 168 (GCA_000009045.1). Alignments were performed using the European Bioinformatics Institute webserver. Phylogenetics analysis was conducted with MEGA-X (Kumar et al. 2018). The statistics model used to build the maximum-likelihood tree was GTR+G+I based on the concatenated sequences of *thiO*, *thiS*, *thiG*, and *thiF* of *E. amylovora* and selected plant-associated bacteria.

Biofilm formation assay in vitro and in planta.

In vitro biofilm formation assay was quantified using a crystal violet staining method described previously (Zeng et al. 2013). Briefly, bacterial strains were cultured in 0.5× LB broth in a 24-well plate at 28°C. A glass coverslip was placed in each well at a 30-degree angle. After 48 h, the bacterial culture was removed and 10% crystal violet was added to the wells. The plate was incubated at room temperature for 1 h, following which the glass coverslips were rinsed with water, air dried, and eluted with 200 µl of elution buffer (40% methanol and 10% glacial acetic acid). The OD₆₀₀ of this elution was measured. The experiment was repeated three times with 12 replicates in each experiment.

The *E. amylovora* WT strain Ea110, Ea110*, Ea110* containing pEA29_{BCN20}, and Ea110* containing both pEA29_{BCN20} and *thiOSGF* construct pJB321-*thiOSGF* (McGhee and Sundin 2008) were individually inoculated into actively growing shoots of 1-year-old potted ‘Gala’ apple trees using the scissor-dip method, as previously described (Koczan et al. 2009). The experiment was conducted twice, with three replicates per experiment. Infected leaves were collected at 7 days postinoculation and the petiole was sectioned into 1-cm sections. Sample fixation in paraformaldehyde/glutaraldehyde, tissue dehydration, critical point drying, and sectioning, mounting, gold coating, and SEM analyses were conducted as described previously (Koczan et al. 2009; Slack et al. 2017).

Amylovoran and cellulose production assays.

The production of amylovoran in supernatants of bacterial cultures was quantified using a turbidity assay with cetylpyrimidinium chloride (CPC) as previously described (Bellemann et al. 1994). Briefly, bacterial cells from overnight LB cultures were

harvested, were washed with PBS, and were inoculated 1:100 into MBMA medium with 1% sorbitol. Cells of $\Delta aceE$ and $\Delta sucA$ mutants were inoculated 1:1 into MBMA medium with 1% sorbitol. Thiamine was applied in the medium when needed. The supernatant of the MBMA culture was tested for the amylovoran concentration by incubating with 50 μ l of CPC (50 mg/ml) per milliliter of supernatant sample for 10 min. The resulting turbidity was measured at OD₆₀₀ and normalized to the OD₆₀₀ values of the cultures grown in MBMA medium.

Production of cellulose in *E. amylovora* was assessed as described (Castiblanco and Sundin 2018). Bacterial strains were spot-inoculated onto LB medium lacking sodium chloride and were amended with Congo red (40 μ g/ml) and Coomassie brilliant blue (20 μ g/ml). Plates were then incubated at 28°C for 24 h. Redness of colonies indicating the production of cellulose was evaluated visually. The experiments were repeated three times with three biological replicates in each experiment.

Levansucrase activity assay and bacterial morphology of *E. amylovora* on MM-T.

Levansucrase activity was quantified as described previously (Hildebrand et al. 2006). Briefly, supernatant of bacterial culture was mixed with 2 M sucrose in PBS in a 1:1 ratio. The mixture was then incubated with shaking at 28°C for 24 h and the resulting turbidity was measured at OD₆₀₀. Levansucrase activity was reported by normalizing the OD₆₀₀ values of turbidity to the OD₆₀₀ of the bacterial culture.

Bacterial morphologies were assessed from strains grown on MM-T plates. Overnight bacterial cultures in LB (5 μ l; OD₆₀₀ = 0.8) were spotted onto MM-T plates. Petri plates were then incubated at 28°C and were photographed daily. The experiments were repeated three times with three biological replicates in each experiment.

Carbohydrate analysis of EPS.

E. amylovora strains Ea110, Ea110*, and Ea110*/pEA29_{BCN20} were grown in 1 liter of MM-T broth for 48 (Ea110) or 96 h (Ea110* and Ea110*/pEA29_{BCN20}) at 28°C. Bacterial cells were removed by centrifugation at 16,000 $\times$ g for 20 min at 4°C. The supernatant was passed through a 1.2- μ m filter, followed by a 0.8- μ m filter, prior to addition of 95% ethanol to a final concentration of 70% (vol/vol). This solution was then stored overnight at 4°C to precipitate EPS. Precipitated EPS was recovered by centrifugation at 16,000 $\times$ g for 20 min at 4°C and was resuspended in 170 ml of PBS (8.0 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ per liter; pH 7.2 to 7.4). Ethanol precipitation and centrifugation were repeated once, followed by resuspension of the precipitated EPS in 30 ml of 0.1 M EDTA, pH 7.0. This EPS suspension was then transferred to prepared dialysis tubing and was dialyzed against deionized water at 4°C with continuous stirring overnight. Dialyzed material was then transferred from the tubing to a 50-ml conical tube and the tube was covered with four layers of cheesecloth. The conical tube was then placed at -80°C for 5 h, prior to lyophilization overnight. Ten milligrams of lyophilized EPS were used for glycosyl composition analysis.

Glycosyl composition analysis was performed at the Complex Carbohydrate Research Center, University of Georgia (Athens, GA, U.S.A.) using previously described methods (Merkle and Poppe 1994; York et al. 1986). In brief, the analysis was done by combined GC-MS of the per-*O*-trimethylsilyl (TMS) derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. Methyl glycosides were first prepared from the dry sample by methanolysis in 1 M HCl in methanol at 80°C (18-22 h), followed by re-*N*-acetylation with pyridine and acetic anhydride in methanol (for detection of amino sugars). The sample was then per-*O*-trimethylsilylated by treatment with Tri-Sil (Pierce) at 80°C (0.5 h).

Table 2. Bacterial strains and plasmids used in this study

Strains and plasmids	Relevant characteristics ^a	Reference or source
<i>Erwinia amylovora</i>		
Ea110	Wild type	Ritchie and Klos 1977
Ea1189	Wild type	Zhao et al. 2009
Ea110*	pEA29-cured Ea110	McGhee and Sundin 2008
BCN20	Wild type, Tn5393 on pEA29	McGhee and Jones 2000
Ea1189 Δ ugd	Δ ugd::Km; Cm ^r , EAM_1917 deletion mutant in Ea1189	This study
Ea1189 Δ sucA	Δ sucA::Km; Km ^r , EAM_1172 deletion mutant in Ea1189	This study
Ea1189 Δ aceE	Δ aceE::Km; Km ^r , EAM_0747 deletion mutant in Ea1189	This study
Ea1189 Δ thiCE	Δ thiC Δ thiE::Km; Km ^r , EAM_0239 and EAM_0240 deletion mutant in Ea1189	This study
Ea1189 Δ thiMD	Δ thiM Δ thiD::Km; Km ^r , EAM_2192 and EAM_2193 deletion mutant in Ea1189	This study
Ea1189 Δ thiI	Δ thiI::Km; Km ^r , EAM_0976 deletion mutant in Ea1189	This study
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> Δ lacU169 (ϕ 80lacZ Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Lab stock
Plasmids		
pKD4	Template plasmid for kanamycin cassette, Km ^r	Datsenko and Wanner 2000
pKD3	Template plasmid for chloramphenicol cassette, Cm ^r	Datsenko and Wanner 2000
pKD46	Arabinose-inducible lambda red recombinase, Ap ^r	Datsenko and Wanner 2000
pPROBE-NT	Promoter-probe vector, promoterless <i>gfp</i> , Ap ^r	Miller et al. 2000
pNT- <i>hrpA</i>	pPROBE-NT containing putative <i>hrpA</i> promoter- <i>gfp</i> transcriptional fusion, Ap ^r	This study
pNT- <i>hrpN</i>	pPROBE-NT containing putative <i>hrpN</i> promoter- <i>gfp</i> transcriptional fusion, Ap ^r	This study
pNT- <i>hrpL</i>	pPROBE-NT containing putative <i>hrpL</i> promoter- <i>gfp</i> transcriptional fusion, Ap ^r	This study
pNT- <i>amsG</i>	pPROBE-NT containing putative <i>amsG</i> promoter- <i>gfp</i> transcriptional fusion, Ap ^r	Peng et al. 2021
pBBR1-MCS5	Broad-host-range plasmid, Gm ^r	Kovach et al. 1995
pBBR1- <i>ugd</i>	<i>ugd</i> cloned in pBBR1-MCS5, Gm ^r	This study
pBBR1- <i>sucA</i>	<i>sucA</i> cloned in pBBR1-MCS5, Gm ^r	This study
pBBR1- <i>aceE</i>	<i>aceE</i> cloned in pBBR1-MCS5, Gm ^r	This study
pBBR1- <i>thiCE</i>	<i>thiCE</i> cloned in pBBR1-MCS5, Gm ^r	This study
pBBR1- <i>thiI</i>	<i>thiI</i> cloned in pBBR1-MCS5, Gm ^r	This study
pBBR1- <i>thiMD</i>	<i>thiMD</i> cloned in pBBR1-MCS5, Gm ^r	This study
pJB321	Cloning vector, Ap ^r	Blatney et al. 1997
pJB321- <i>thiOSGF</i>	<i>thiOSGF</i> cloned in pJB321, also known as pGCM17, Ap ^r	McGhee and Sundin 2008

^a Ap^r, Cm^r, Km^r, and Gm^r = ampicillin, chloramphenicol, kanamycin, and gentamicin resistance.

Transcriptional activity assay using a green fluorescent protein (GFP) reporter plasmid.

The promoter regions of T3SS regulon genes and *amsG* were cloned into the promoter probe vector pPROBE-NT, which contains a ribosomal binding site upstream of the *gfp* gene (Leveau and Lindow 2001; Miller et al. 2000), respectively. The resulting plasmids were electroporated into *E. amylovora*. Bacterial cells containing reporter plasmid pNT-*hrpA*, pNT-*hrpN*, or pNT-*hrpL* were grown in LB medium and were then inoculated 1:100 into Hrp-inducing MM at 28°C for 16 h. Cells containing pNT-*amsG* were first grown in LB medium and were then inoculated 1:100 into MBMA medium with 0.1% sorbitol at 28°C for 24 h. The GFP intensities of *E. amylovora* carrying reporter plasmids were measured using the Tecan Spark plate reader (Tecan, Männedorf, Switzerland) with excitement at 488 nm and emission detection at 435 nm. GFP fluorescence was normalized to the OD₆₀₀ of bacterial cultures.

Mutant construction and complementation.

The *thiCE*, *thiDM*, *thiL*, *ugd*, *aceE*, and *sucA* genes or operons were deleted from the genome of *E. amylovora* using the red recombinase method (Datsenko and Wanner 2000). In brief, recombination fragments consisting of 50-nucleotide homology sequences of flanking regions upstream and downstream of each target gene flanking a chloramphenicol or kanamycin cassette were amplified from plasmid pKD3 or pKD4, respectively. PCR products were purified by gel purification and electroporated into *E. amylovora* Ea1189 expressing recombinase genes from the helper plasmid pKD46. Mutants were selected on LB medium amended with chloramphenicol or kanamycin and were further confirmed by PCR, using outside primers and sequencing.

To generate complemented or overexpression strains, the putative promoter and ORF regions of target genes were amplified and cloned into pBBR1-MCS5 or pJB321 (Table 2). The resulting plasmids were then confirmed by sequencing and were electroporated into *E. amylovora* cells.

RNA isolation and quantitative real-time PCR (qRT-PCR).

The mRNA levels of *amsG*, *amsF*, *thiO*, and *thiG* were measured by qRT-PCR. In brief, *E. amylovora* cells cultured in MBMA broth with 1% sorbitol were harvested, and total RNAs were extracted using the RNeasy minikit method (Qiagen, Hilden, Germany), according to manufacturer instructions. DNA was removed using Turbo DNA-free DNase (Ambion, Austin, TX, U.S.A.). cDNA was synthesized using the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, U.S.A.). The cDNA levels of different samples were quantified by RT-PCR using SYBR green PCR master mix (Applied Biosystems, Foster City, CA, U.S.A.). The relative levels of gene expression were determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), with the *recA* gene used as the internal control (Takle et al. 2007). Three technical replicates were used for each target gene.

Growth rate of *E. amylovora* under aerobic and semi-anaerobic conditions.

The growth rate of *E. amylovora* was monitored by measuring OD₆₀₀ values of bacterial cultures in MBMA broth with 0.1% of sorbitol at 28°C with shaking (220 rpm) under an aerobic growth condition or the same liquid cultures layered with mineral oil under semi-anaerobic growth conditions. Two independent experiments were performed with three replicates in each experiment. The OD₆₀₀ values were measured using Tecan Spark plate reader (Tecan).

Statistical analysis.

Means and standard deviations of experimental results were calculated using Excel and the statistical analysis was performed using a two-tailed Student's *t* test (Microsoft, Redmond, WA) or Fisher's least significant difference test with a DPS data processing system.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

DPS processing system: http://www.dpsw.cn/dps_eng
European Bioinformatics Institute webserver: <https://www.ebi.ac.uk>
University of Wisconsin ASAP database:
<https://asap.ahabs.wisc.edu/asap/home.php>

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