Putative transcription antiterminator RfaH contributes to Erwinia amylovora virulence

Sara M. Klee1 | Judith P. Sinn1 | Jeremy Held1,2 | Chad Vosburg1 | Aleah C. Holmes1,3 | Brian L. Lehman4 | Kari A. Peter1,4 | Timothy W. McNellis1

Abstract
The gram-negative bacterium Erwinia amylovora causes fire blight disease of apple and pear trees. The exopolysaccharide amylovoran and lipopolysaccharides are essential E. amylovora virulence factors. Production of amylovoran and lipopolysaccharide is specified in part by genes that are members of long operons. Here, we show that full virulence of E. amylovora in apple fruitlets and tree shoots depends on the predicted transcription antiterminator RfaH. RfaH reduces pausing in the production of long transcripts having an operon polarity suppressor regulatory element within their promoter region. In E. amylovora, only the amylovoran operon and a lipopolysaccharide operon have such regulatory elements within their promoter regions and in the correct orientation. These operons showed dramatically increased polarity in the ΔrfaH mutant compared to the wild type as determined by RNA sequencing. Amylovoran and lipopolysaccharide production in vitro was reduced in rfaH mutants compared to the wild type, which probably contributes to the rfaH mutant virulence phenotype. Furthermore, type VI secretion cluster 1, which contributes to E. amylovora virulence, showed reduced expression in ΔrfaH compared to the wild type, although without an increase in polarity. The data suggest that E. amylovora RfaH directly, specifically, and exclusively suppresses operon polarity in the amylovoran operon and a lipopolysaccharide operon.

Keywords
apple, fire blight, operon, pear, type VI secretion
lipopolysaccharides (LPS) (Piqué et al., 2015). Many of the genes encoding these components are arranged in large operons (Bugert & Geider, 1995; Kim et al., 1997), yielding long, polycistronic transcriptional products.

During an ongoing screen of Tn5 transposon mutants of wild-type E. amylovora strain HKN06P1 (Lee et al., 2010; Table S1) for mutants defective in virulence, two independent mutations were identified in the putative transcriptional antiterminator gene rfaH. The two rfaH mutants have transposon insertions at base positions 296 and 474 of the 498bp rfaH open reading frame, disrupting codons 99 and 158 of 166 predicted codons, respectively (Figure 1a). These mutants were named rfaHTn5-296 and rfaHTn5-474 (Figure 1a). Subsequently, a deletion of the open reading frame of rfaH was constructed (ΔrfaH; Figure 1a). All three rfaH mutants displayed dramatically attenuated fire blight symptom development in an immature apple fruitlet assay (Klee et al., 2019) (Figure 1b). The rfaH mutants grew as well as the wild type in M9 minimal medium with sorbitol (Figure S1), indicating that rfaH disruption did not affect bacterial multiplication or primary metabolism. A copy of rfaH (Figure 1a) cloned into cosmid vector pCE (Figure S2, and Tables S1 and S2) restored the ability of the three rfaH mutants to cause oozing and necrosis in apple fruitlets (Figures 1c and S3). Fire blight symptoms caused by rfaHTn5-474 in apple tree branches inoculated by shoot tip wounding (Klee et al., 2020) were significantly reduced and delayed compared to the wild type (Figure 1d). At each time point, the complementation strain caused significantly more severe disease than the rfaH mutant. Together, these results indicate that rfaH is essential for full virulence of E. amylovora.

In prokaryotes, the uncoupling of transcription and translation can lead to rho-dependent termination of transcripts, particularly in long polycistronic mRNAs. This causes operon polarity, in which promoter-distal genes have decreased expression compared to
promoter-proximal genes (Bailey et al., 1996). Long operons responsible for the synthesis of EPS, LPS, haemolysins and sex factors may have a short, conserved sequence called an operon polarity suppressor (ops) in the 5’ untranslated region (5’ UTR) (Bailey et al., 1997; Nagy et al., 2002; Nieto et al., 1996). ops mutations trigger increased operon polarity, and overexpression of RfaH suppresses this effect (Bailey et al., 1996). RfaH reduces pausing at both rho-dependent and rho-independent termination sites (Belogurov et al., 2009) during its interaction with the transcription elongation complex paused at ops sequences on the non-template strand of DNA (Artsimovitch & Landick, 2002).

RfaH is a paralog of the universal transcriptional regulator NusG. RfaH successfully competes with the greater abundance of NusG by binding more tightly than NusG to RNA polymerase (Wang & Artsimovitch, 2021). RfaH-modified RNA polymerases move at a rate about three times faster than unmodified polymerases and may act to overcome the natural pauses in transcribing long operons (Artsimovitch & Landick, 2002). The bulk of current knowledge about the ops and rfaH function comes from studies with animal pathogens such as Escherichia coli and Salmonella enterica.

Geneious Prime was used to search for all permutations of the ops consensus sequence GCGGTAAGnTG (Artsimovitch & Landick, 2000) in a draft genome of wild-type E. amylovora strain HKN06P1 (unpublished data); the identical candidates were also present in the E. amylovora CFBP 1430 reference genome (Smits et al., 2010). Only three ops elements were detected that were in the correct orientation relative to gene transcription and located in a predicted 5’ UTR (Table 1). These ops elements were upstream of the rfb LPS operon, the ams amyllovoran operon, and yhbY, a 294 bp stand-alone gene predicted to encode ribosome assembly protein YhbY (Table 1).

To examine effects of deletion of rfaH on gene expression, transcriptomes of wild-type and ΔrfaH bacteria were compared using RNA-sequencing (RNA-Seq; Methods S1). Three independent cultures of each strain were grown to mid-log phase in amylovoran minimal medium and RNA was isolated as previously described (Klee et al., 2020). Following rRNA depletion, single-end, 75 bp RNA-Seq was performed at the Penn State Genomics Core Facility on an Illumina NextSeq 550. Differential expression analysis was performed in Geneious Prime using the DESeq2 method (Love et al., 2014).

The DESeq2 analysis of the transcriptome data identified 58 genes with a log2 fold change (LFC) >1 or <−1 and an adjusted p value <0.01 (Figure S4). Of these 58 genes, 52 were expressed at a lower level in ΔrfaH relative to the wild type, while only six were expressed at a higher level (Figure S4 and Table S3). The 58 differentially expressed genes identified in our analysis represent c.1.5% of genes in E. amylovora, which is in line with rfaH’s regulatory action on a small subset of genes in animal pathogens (Wang & Artsimovitch, 2021). Principal component analysis (PCA) of the expression data showed clear clustering of the wild-type and ΔrfaH samples along the first principal component (PC1; Figure S5), supporting the validity of the results. Analysis of RNA-Seq reads showed that the rfaH promoter was still active in ΔrfaH and produced a short transcript without a coding region (Figure S6).

Genes in the ams operon, which is involved in amyllovoran production (Bugert & Geider, 1995), had reduced transcript abundance in ΔrfaH compared to the wild type, with LFCs ranging from −1 to −4 (Figure 2a and Table S3). Genes in the rfb operon, which is involved in LPS and EPS production (Klee et al., 2020), also had reduced transcript abundance in ΔrfaH relative to the wild type, with LFCs from −1.8 to −5.3 (Figure 2b and Table S3). In both the rfb and ams clusters, ops-distal genes tended to be more down-regulated than ops-proximal genes (Figure 2a,b). These gene expression results and the presence of ops elements in the promoter regions of both operons (Figure 2a,b and Table 1) are consistent with the operons’ dependence on putative transcription antiterminator activity of RfaH for normal expression. Although yhbY has a predicted ops (Table 1), yhbY was not differentially expressed in ΔrfaH compared to the wild type (Table S3). Therefore, we conclude that yhbY expression is not directly or indirectly influenced by RfaH.

Although type VI secretion system gene cluster 1 (T6SS-1; De Maayer et al., 2011) does not have a predicted ops, genes in this cluster had reduced transcript abundance in ΔrfaH compared to the wild type across the cluster, with LFCs ranging between −1.5 and −4.8. There was no consistent pattern of increased down-regulation of distal genes relative to the genes proximate to the start of the gene cluster (Figure 2c and Table S3). T6SS-1 does not have an ops sequence upstream, suggesting that RfaH is not involved in polarity suppression in the T6SS-1 gene cluster. No differential gene expression between ΔrfaH and the wild type was detected in the other two T6SS clusters (T6SS-2 and T6SS-3; Table S3).

Table 1: Locations of predicted ops elements in relation to potential regulatory target genes

| Ops location | Ops sequence (5’–3’) | Potential ops regulatory target gene or gene cluster | Gene cluster or single gene open reading frame span | Number of genes |
|--------------|-----------------------|----------------------------------------------------|-----------------------------------------------|----------------|
| 2,296,419 to 2,296,430 [complement] | GCGGTAAGcTG | ams gene cluster (amyllovoran) | 2,280,397 to 2,296,193 [complement] | 12 |
| 2,277,972 to 2,277,983 [complement] | GCGGTAAGcTG | rfb gene cluster (LPS or EPS) | 2,268,192 to 2,277,963 [complement] | 9 |
| 395,731 to 395,742 [complement] | GCGGTAAGaTG | yhbY gene (ribosome assembly RNA-binding protein YhbY) | 395,402 to 395,695 [complement] | 1 |

Note: Nucleotide positions are in the Erwinia amylovora CFBP 1430 reference genome, GenBank accession FJ434113.1 (Smits et al., 2010).
RNA-Seq coverage maps illustrate the sharp increase in polarity in the rfb and amS gene clusters in ΔrfaH compared to the wild type (Figure 3a). The method of Johnson et al. (2020) was used to quantify operon polarity for these as well as several other gene clusters and monocistronic transcripts for comparison (Figure 3b). Polarity is represented as a ratio of the mRNA read density at the

**FIGURE 2** Effect of deletion of rfaH on expression of the *Erwinia amylovora* amylovoran amS and lipopolysaccharide rfb operons and type VI secretion system cluster 1 (T6SS-1), as determined by RNA sequencing. (a) Relative transcript abundance for amS operon genes in ΔrfaH compared to the wild type. The ops element position is indicated. (b) Relative transcript abundance for rfb operon genes in ΔrfaH compared to the wild type. The ops element position is indicated. (c) Relative transcript abundance for T6SS-1 genes in ΔrfaH compared to the wild type. For clarity, the commonly used *E. amylovora* locus names as given in GenBank annotation FN434113.1 are used wherever available; locus names or numbers from NCBI reference annotation NC_013961.1 are used for all other loci.
FIGURE 3 Deletion of rfaH exacerbates 5′ operon polarity in the Erwinia amylovora ams and rfb gene clusters. (a) RNA-Seq coverage (reads per base) maps of the indicated gene clusters in wild-type and ΔrfaH E. amylovora. (b) 5′ polarity of the indicated genes and gene clusters in wild-type and ΔrfaH E. amylovora. Operon polarity was calculated as the ratio of mRNA reads mapping to the 5′-most 15% (“start”) of the gene or gene cluster to reads mapping to the 3′-most 15% (“end”) gene regions indicated in panel (a). Asterisks indicate statistically significant differences by t test (p ≤ 0.01, n = 3) comparison. n.s., not significant; kb, kilobase pairs.
beginning and end of the gene cluster, where values greater than 1 indicate 5′ polarity. While the rfb and ams gene clusters in the wild type displayed some degree of 5′ polarity, the polarity of both clusters was significantly higher in the ΔrfaH mutant compared to the wild type. These trends are consistent with an antiterminator role for RfaH in rfb and ams gene cluster transcription.

While the T6SS-1 gene cluster also exhibited some degree of 5′ polarity in the wild type, 5′ polarity was significantly decreased in ΔrfaH compared to the wild type (Figure 3b). Thus, RfaH does not appear to play an antitermination role for the T6SS-1 gene cluster, which is consistent with the lack of an ops element upstream of this gene cluster. The RNA-Seq coverage map for T6SS-1 showed a generally similar pattern in the wild type and ΔrfaH, with dramatically reduced numbers of reads across the entire T6SS-1 gene cluster in ΔrfaH (Figure 3a). The decreased polarity of the T6SS-1 gene cluster in ΔrfaH compared to the wild type is explained by the peak in RNA-Seq reads in the “end” region of the T6SS-1 cluster, which results in a substantially greater number of RNA-Seq reads in the end region compared to the “start” region (Figure 3a).

To put the polarity analysis into a wider context, polarity was analysed for one additional presumptive operon and two representative genes. The iron sulphur cluster assembly presumptive 5.7 kb operon (sufA–sufE) had no difference in 5′ polarity in ΔrfaH compared to the wild type (Figure 3). The same was true for putative helicase-encoding gene RS28070 (Figure 3) and the virulence gene hrpN (Wei et al., 1992; Figure S7). The hrpN gene was selected because of its strong baseline expression and average gene length for E. amylovora, while RS28070 was selected because it has a long single open reading frame of 5.5 kb. The polarity analysis quantifies the antitermination effect of RfaH on the rfb and ams gene clusters in E. amylovora and emphasizes the specificity of this activity.

The gene expression changes in the ams operon in the ΔrfaH mutant compared to the wild type (Figure 2a) suggest that amylovoran production might be affected by loss of rfaH. Amylovoran levels were indeed significantly lower in the ΔrfaH mutant compared to the wild type (Figure 4a). Because amylovoran is important for E. amylovora virulence (Bellemann & Geider, 1992; Bennett & Billing, 1978),

![Figure 4](https://example.com/figure4.png)

**FIGURE 4** Virulence-related phenotypes of Erwinia amylovora rfaH mutants. (a) Amylovoran production of indicated strains after 24h growth in amylovoran minimal medium. Asterisks indicate a statistically significant difference from the wild type by pairwise t tests (p ≤ 0.01, n = 3). (b) Lipopolysaccharide (LPS) production by indicated strains as visualized by polyacrylamide gel electrophoresis and silver staining. (c) Hypersensitive response elicitation by indicated strains infiltrated at 1 × 10⁸ cfu/ml into Nicotiana tabacum ‘Glurk’, photographed 48h after infiltration. Four leaves were inoculated for this experiment, with all four leaves showing identical results. (d) Swimming motility in 0.3% Luria Bertani agar at 36 h postinoculation with indicated strains; the Mot− negative control strain is a nonmotile Tn5 mutant of HNK06P1. Average diameter of swimming area indicated with standard deviations; the asterisk indicates the statistically significant difference from the wild type by pairwise t test (p ≤ 0.01, n = 5) comparison. Each assay was performed at least twice with the same result.
lower production of this EPS probably contributes to rfaH mutants’ virulence defect. In contrast, the stand-alone lsc gene, encoding the levansucrase enzyme responsible for synthesis of the EPS levan, was not differentially expressed in the rfaH mutant compared to the wild type (Table S3), and levansucrase enzymatic activity was unaffected by mutation of rfaH (Figure S8).

LPS production was also altered in the rfaH mutants (Figure 4b), consistent with reduced expression of the rfb operon (Figure 2b). LPS contributes to bacterial survival in hosts (Kutscher & Ranf, 2019) and virulence is reduced in E. amylovora strains lacking O-antigen (Berry et al., 2009; Ray et al., 1986). Our results show that rfaH is required by E. amylovora for normal LPS and EPS production, as reported for gram-negative animal pathogens (Bittner et al., 2002; Garrett et al., 2016; Leskinen et al., 2015; Nagy et al., 2002).

Several genes in the T6SS-1 gene cluster result in reduced pathogenicity when deleted in E. amylovora (Tian et al., 2017). The significantly reduced expression of these genes in ΔrfaH might contribute to the virulence defect of ΔrfaH. In addition, altered amylovoran levels have been reported in T6SS gene deletion mutants of E. amylovora (Tian et al., 2017). The significantly decreased expression of these T6SS-1 genes in ΔrfaH compared to the wild type could potentially contribute to the reduced amylovoran production in ΔrfaH compared to the wild type. Deletion analysis of T6SS genes has also demonstrated their importance in E. amylovora competition with other bacteria (Kamber et al., 2017; Tian et al., 2017). However, we found no consistent difference in ΔrfaH and the wild type’s ability to compete with E. coli during in vitro qualitative (Figure S9) and quantitative (Figure S10) competition assays (Decoin et al., 2015; Tian et al., 2017). It might be expected that T6SS gene deletions would result in stronger phenotypic changes than the relatively reduced gene expression across the T6SS-1 cluster we detected in ΔrfaH.

E. amylovora causes a T3SS-dependent hypersensitive response (HR) when infiltrated into leaves of nonhost tobacco (Nicotiana tabacum). While several long presumptive operons are involved in E. amylovora T3SS production (Kim et al., 1997; Oh et al., 2005), no ops elements reside upstream of these operons, predicting that rfaH is not involved in their regulation. This was confirmed by the RNA-Seq data, which showed that transcript abundance of T3SS genes did not differ between ΔrfaH and the wild type (Table S3). Consistent with this finding, rfaH mutants triggered an HR in tobacco leaves, as did the wild type (Figure 4c).

Swimming motility contributes to E. amylovora virulence in natural infections (Bayot & Ries, 1986), and reduced swimming motility due to lack of antiterminator activity has been observed in other pathogens, such as S. enterica (Kong et al., 2009). While several predicted swimming motility-associated genes, including flgB1, flgC1 and flgD1, had lower transcript abundance in ΔrfaH compared to the wild type, the data did not meet our fold-change cut-off (Table S3). ΔrfaH consistently showed a slight, though not statistically significant, reduction in swimming motility (Figure 4d). Thus, decreased expression of certain flagellin genes in ΔrfaH was insufficient to have a major impact on swimming motility. Because ops elements were not found upstream of these motility-associated genes, the relatively minor effects on motility gene expression and phenotype appear to be indirect results of rfaH deletion.

Our results support the conclusion that the rfaH gene in E. amylovora encodes a transcriptional antiterminator, as predicted by the genome annotation. We also conclude that E. amylovora RfaH directly and specifically influences the transcription of the rfa and rfb operons in E. amylovora to facilitate normal amylovoran and LPS production and full virulence. We surmise that RfaH effects on T6SS-1 expression are indirect, possibly resulting from the alterations in amylovoran and LPS in ΔrfaH. Interestingly, while other long operons and gene clusters are required for E. amylovora virulence, including T3SS gene clusters, these do not appear to require the antiterminator activity of RfaH for normal expression. Our data indicate that RfaH transcription antitermination influence is restricted to the rfa and rfb operons in E. amylovora. The substantially attenuated virulence of E. amylovora rfaH mutants is consistent with similar findings for rfaH mutants in animal pathogens including S. enterica, K. pneumoniae, and E. coli (Bachman et al., 2015; Gao et al., 2013; Nagy et al., 2006). While an ops element was noted upstream of a putative EPS operon in Dickeya dadanilii (Erwinia chrysanthemi); Condemine et al., 1999), we have not discovered any other examinations of RfaH function in plant pathogens.

**AUTHOR CONTRIBUTIONS**

S.M.K., J.P.S., J.H., C.V., A.C.H., B.L.L. and K.A.P. planned and performed experiments and collected data. T.W.M. planned and coordinated the study. S.M.K., J.P.S., J.H. and T.W.M. wrote the manuscript. All the co-authors reviewed, edited and approved the manuscript.

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**DATA AVAILABILITY STATEMENT**

The RNA-seq data will be deposited in a MIAME-compliant database upon acceptance for publication.

**ORCID**

Timothy W. McNellis https://orcid.org/0000-0002-8764-900X
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