TfmR, a novel TetR-family transcriptional regulator, modulates the virulence of Xanthomonas citri in response to fatty acids

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SUMMARY

The type III secretion system (T3SS) is required for Xanthomonas citri subsp. citri (Xcc) virulence by translocating effectors into host cytoplasm to promote disease development. The T3SS is controlled by the master transcriptional regulators HrpG and HrpX. While the function of HrpG and HrpX are well characterized, their upstream regulation remains elusive. By using transposon mutagenesis, we identified XAC3052, a TetR-family transcriptional regulator, which regulates T3SS gene expression. Deletion of XAC3052 caused significant reduction in the expression of T3SS and effector genes in vitro and in planta; as well as reduction of virulence in sweet orange (Citrus sinensis). Overexpression of hrpG restored the virulence of ΔXAC3052, suggesting that the loss of virulence is caused by reduction of T3SS gene expression. XAC3052 directly binds to the promoter region and represses the transcription of fadE, mhpC and fadH genes. FadE, MhpC and FadH are not involved in T3SS regulation, but involved in fatty acid catabolism. ΔXAC3052 displays altered fatty acid composition and retarded growth in environments limited in fatty acids. Exogenously supplemented long-chain fatty acids activate the fadEl/mhpC promoter and suppress T3SS promoters in wild-type Xac but not in ΔXAC3052. Moreover, the binding of XAC3052 to its target promoter was disrupted by long-chain fatty acids in vitro. Herein, XAC3052 is designated as Tfmr (T3SS and Fatty acid Mechanism Regulator). This study identifies a novel regulator of fatty acid metabolism and suggests that fatty acids play an important role in the metabolic control of virulence in Xcc.

Keywords: fatty acids, pathogenicity, TetR, type III secretion system, Xanthomonas citri.

INTRODUCTION

Xanthomonas pathogens utilize the type III secretion system to deliver effector proteins to manipulate host signalling to promote disease (Büttnner and Bonas, 2010). Regulation of the type III secretion system (T3SS) and type III effector genes in Xanthomonas is dependent on the two master regulators HrpG and HrpX (Guo et al., 2011; Wengelnik and Bonas, 1996; Wengelnik et al., 1996). The OmpR family transcriptional regulator HrpG is induced during early infection (Potnis et al., 2015) and promotes the expression of the AraC-type transcriptional activator hrpX, which in turn positively regulates the expression of the T3SS structural components and type III effector genes by binding to a conserved sequence motif (plant-inducible promoter [PIP] box) in the promoter regions of these genes (Koebnik et al., 2006; Kogenaru et al., 2012; Wengelnik and Bonas, 1996). HrpG and HrpX are subjected to complex regulation through transcription (Kametani-Ikawa et al., 2011; Lu et al., 2011), transcript stability (Andrade et al., 2014), protein stability (Zhou et al., 2018) and post-transcriptional modification (Li et al., 2014), and are under tight metabolic control (Rashid et al., 2016; Schulte, 1992). While many advances have been made in understanding the upstream regulation of HrpG and HrpX, the mechanism that enables the selective induction and activation of these proteins remains unclear.

TetR-family regulators (TFRs) are a large family of bacterial transcriptional regulators (Ramos et al., 2005). Their regulatory function greatly varies and was found to be involved in multidrug resistance, catabolism, osmotic stress, quorum sensing, antibiotic production and host response (Ramos et al., 2005). TFRs typically function as transcriptional repressors that control the expression of their target genes in response to ligand molecules. All TFRs consist of an N-terminal helix-turn-helix DNA binding domain and a C-terminal domain that functions in dimerization and ligand interaction (Cuthbertson and Nodwell, 2013; Deng et al., 2013). In the absence of ligand, TFR homodimers bind to a palindromic operator sequence in their perspective target promoters and inhibit their transcription (Deng et al., 2013). In the presence of ligand, the ligand directly binds to the regulator and promotes a conformational

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change that releases the TFR from its target promoter and enables its transcription (Deng et al., 2013). The sequence or the operator, characteristic of the ligand and the number of target genes are unique to each TFR and vary greatly. In many cases, TFRs are located in small transcriptional units in divergent orientation to their target genes (Ramos et al., 2005).

In bacteria, long-chain fatty acids are catabolized into acetyl-CoA via the beta-oxidation pathway and mediated by fatty acid degradation (Fad) enzymes (Fujita et al., 2007). The beta-oxidation pathway is highly conserved but its regulation differs significantly and is controlled by TFRs in some bacteria: YsiA of Bacillus subtilis functions as a master regulator of Fad by controlling multiple beta-oxidation coding genes in dependence of long-chain fatty acyl-coAs (Fujihashi et al., 2013; Matsuoka et al., 2007), PstA of Pseudomonas aeruginosa controls the fadB/A operon and fadE in dependence of long-chain fatty acids (Kang et al., 2008; Wells et al., 2017); and Thermus thermophiles FadR controls multiple beta-oxidation related operons (Agari et al., 2011).

Most fatty acid related research in Xanthomonas is focused on the diffusible signal factor (DSF), a fatty acid derivative that acts as the main quorum sensing molecule (Guo et al., 2012; Li et al., 2019; Zhou et al., 2017a). Numerous studies were conducted regarding the metabolic control of DSF production and some have directly linked the production and sensing of DSF to fatty acid biosynthesis and beta-oxidation related genes (Bi et al., 2014; Yu et al., 2016). However, very few studies have investigated the utilization of fatty acids as an energy source and regulation of the beta-oxidation pathway itself and its relationship to virulence.

In this study, we identified the TFR protein TfmR as a novel virulence regulator in Xanthomonas citri subsp. citri, the causal agent of citrus canker (Vojnov et al., 2010). We found that TfmR acts as an indirect positive regulator of the T3SS and type III effector genes and a direct negative regulator of fatty acid catabolic genes, fadE, mphC and fadH, in response to long-chain fatty acids. We demonstrated that supplementing long-chain fatty acids to T3SS inducing media suppresses the expression of virulence genes in dependence of TfmR. We provide novel insights into the regulation of fatty acid metabolism and the relationship between fatty acid homeostasis and virulence regulation in Xanthomonas.

RESULTS

TfmR is required for pathogenicity of Xcc

We conducted random mutagenesis using EZ-Tn5 transposon (Epicentre) to identify novel regulators of T3SS in Xcc. The Tn5 transposon was transformed into Xcc 306 strain that harbours a plasmid expressing β-glucuronidase (GUS) under the control of the promoter region of the T3S translocon hrpF (Locus tag XAC0394). Clones that displayed reduction in GUS activity were tested for virulence in sweet orange and the Tn5 insertion site was determined in 16 clones that displayed reduced virulence (Fig. S1 and Table S1). Two independent Tn5 mutants identified during the EZ-Tn5 transposon screen were mutated in TfmR (Locus tag XAC0352; Table S1, Fig. S2A and B) that encodes a TetR-family transcriptional regulator (Fig. S2C). Homology search has identified that TfmR is conserved in all Xanthomonas spp. and its homologs are present in the majority of bacteria in the
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Xanthomonadaceae order but not in other bacteria outside of Xanthomonadaceae (Fig. S2C).

To investigate whether the reduction in virulence in the TfmR Tn5 mutants is caused by disruption of *tfmR*, we generated a deletion mutant of *tfmR* (*XccΔtfmR*, Fig. S2A and D). Similar to the Tn5 mutants, sweet orange leaves infected with *XccΔtfmR* displayed less disease symptoms compared to that infected with the wild-type *Xcc* (Fig. 1A). Additionally, *XccΔtfmR* bacterial population in sweet orange leaves was significantly reduced compared to the wild-type *Xcc* at 3, 6 and 9 days post-inoculation (dpi) (Fig. 1B). Reintroduction of *tfmR* gene into *XccΔtfmR* by plasmid restored canker symptoms and bacterial population to the wild-type level (Fig. 1A and B). To further characterize the role of *tfmR* in bacterial pathogenesis we monitored multiple virulence associated traits. *XccΔtfmR* showed significant reduction in motility and production of exopolysaccharides (EPS) (Fig. 1C, D and S3A). On the other hand, *XccΔtfmR* did not affect hydrolase and catalase activity or resistance to exogenous SDS or hydrogen peroxide (Fig. S3).

TfmR regulates T3SS and effector gene expression

The pathogenicity of *Xcc* is highly dependent on the delivery of T3Es via the T3SS (Yan and Wang, 2011; White et al., 2009). Since
and Xcc∆tmfR were transformed with a series of reporter plasmids expressing GUS under the control of the promoter regions of hrpX, xopAU and hrpF but not that of gyrA (XAC1631) and the T3SS translocon hrpF. To eliminate the possibility that Xcc∆tmfR may demonstrate reduction in transcription or in the stability of the GUS protein, Xcc∆tmfR was also introduced with a reporter plasmid expressing GUS under the control of the DNA gyrase subunit A (gyrA, XAC1631) promoter. When grown in a T3SS inducing medium, XVM2, Xcc∆tmfR displayed significant (P-value < 0.05) reduction in GUS activity under the control of the promoters of hrpX, xopAU and hrpF compared to Xcc wild type and Xcc∆tmfR complemented strain (Fig. 2C and D), indicating that TfmR contributes to T3SS gene expression and effector translocation in planta. We previously reported that T3SS gene expression is required for Xcc induction of hypersensitive response (HR) in N. benthamiana (Zhou et al., 2015, 2017b). Xcc cells were infiltrated into leaves of N. benthamiana and HR-like cell death was monitored visually and by quantification of ion leakage. Leaves infected with Xcc∆tmfR or Xcc hrcV:Tn5, a mutant in one of the structural genes of type III secretion system apparatus (Fenselau et al., 1992), developed weakened and delayed cell death compared to Xcc wild type and Xcc∆tmfR complemented strain (Fig. 2E and F), thus further establishing the contribution of TfmR to T3SS translocation in planta. The reduction of virulence of Xcc∆tmfR in citrus may result from the reduction in the expression of T3SS and effector genes in planta or through other mechanisms. To examine if T3SS expression is responsible for this phenotype in planta, we introduced to Xcc∆tmfR a plasmid, which constitutively expressed the T3SS master regulator HrpG under the lac promoter. We then tested whether the constitutive expression of HrpG can bypass the requirement of tmfR for bacterial growth and symptom development. Constitutive expression of HrpG in Xcc∆tmfR restored symptom development and bacterial growth in planta (Fig. 3A and B). This indicates that the virulence reduction in Xcc∆tmfR possibly results from the decreased expression of T3SS genes.

**TfmR does not bind to the promoter of hrpG and hrpX directly**

TetR proteins have been known to regulate target genes by binding to their promoter regions with their characteristic helix-turn-helix (HTH) DNA binding motif (Ramos et al., 2005). We therefore hypothesized that TfmR might control the T3SS and T3Es by directly controlling the expression of the T3SS master regulators hrpG or hrpX. We tested direct interaction between TfmR and the promoter regions of hrpX and hrpG in vitro and in vivo by electrophoretic mobility shift assay (EMS) and Chromatin Immunoprecipitation-Quantitative Polymerase Chain Reaction.

**Fig. 3** Complementation of Xcc∆tmfR by overexpression of HrpG. Sweet orange leaves were syringe-infiltrated with suspensions (1 × 10⁸ CFU/mL for A, 1 × 10⁷ CFU/mL for B) of Xcc wild type (WT), Xcc∆tmfR, Xcc∆tmfR + tmfR, and Xcc∆tmfR carrying a plasmid expressing hrpG under the control of a lac promoter (+ OE hrpG). (A) Inoculated leaf was photographed at 5 days post-inoculation. The experiment was repeated three times with similar results. Letters denote significant differences based on analysis of variance (ANOVA) and comparisons for all pairs using Student’s t-Test (P-value < 0.05).
(ChIP-qPCR) (Kim and Dekker, 2018). For EMSA analysis the 467 bp, 699 bp and 189 bp sequences upstream of the start codons of hrpX, hrpG and gyrA (DNA gyrase gene, used as a negative control), respectively, were used as probes and incubated with purified TfmR protein fused to Glutathione S-transferase (GST) tag. No interaction was observed between GST-TfmR and the hrpX, hrpG or the gyrA promoters (Fig. S4A). ChIP-qPCR analysis was conducted by purifying TfmR fused to hemagglutinin (HA) from Xcc and quantifying the relative abundance of the intragenic region between hrpX and hrpG in the purified samples by qRT-PCR. Similarly to the in vitro analysis, we did not observe direct interaction between TfmR and the hrpG/hrpX promoters in vivo (Fig. S4B). This indicates that the effect of TfmR on the transcription of T3SS and T3E genes is likely to be indirect.

**TfmR is a transcriptional repressor of the mhpC/fadE operon**

The majority of transcriptional regulators of the TetR-family are found in a divergent orientation to a neighbouring gene, which is directly regulated by them and many also regulate their own transcription (Cuthbertson and Nodwell, 2013). Upon examination of the available genome sequence deposits of bacteria from the Xanthomonadaceae order in the Integrated Microbial Genomes and Microbiomes database (https://img.jgi.doe.gov/), we identified that tfmR and its homologs in different Xanthomonadaceae are found in an opposite orientation to an operon encoding fadE (XAC3054 in Xcc) and mhpC/bioH family methyl ester carboxylesterase (XAC3053 in Xcc, absent in X. albilineans, Lysobacter and Luteimonas) (Fig. 4A). Therefore, we hypothesized that this operon is under the control of TfmR. To test this, a GUS reporter was placed under the control of the promoter region of XAC3053/XAC3054 (p mhpC/ade) and TfmR (p tfmR) (Fig. 4B), and the GUS activity from each promoter was tested in Xcc wild type and XccΔtfmR. No difference was observed in the promoter activity of ptfmR, whereas the promoter activity of pmhpC/ade was 50- to 100-fold higher in XccΔtfmR compared to the wild type (Fig. 4B). Up-regulation of both mhpC and ade was also observed in XccΔtfmR by qRT-PCR compared to Xcc wild type and the XccΔtfmR complemented strain (Fig. 4C).

**TfmR directly binds to the promoter of the mhpC/fadE operon**

We next tested the direct interaction between TfmR and the promoter regions of mhpC/ade (pmhpC/ade) using EMSA and ChIP-qPCR. Significant shift was observed when GST-TfmR was incubated with pmhpC/ade compared to incubation with GST alone (Figs 5A and S4A). This shift was disrupted by the addition of a competitor, i.e. unlabelled pmhpC/adeDNA, in a dosage dependent manner (Fig. 5A). Direct binding was also observed in vivo using ChIP-qPCR analysis that displayed enrichment of pmhpC/ade in the TfmR-HA purified samples (Fig. S4B). We characterized the affinity of GST-TfmR to pmhpC/ade DNA probe using decreasing protein concentrations and the KD value was determined to be 86.97 ± 21.83 nM (Fig. 5B). This indicates that the effect of TfmR on the transcription of its divergent
neighbouring genes is based on direct interaction with the corresponding promoter sequences.

We next examined if the virulence reduction observed in Xcc∆tfmR is dependent on the pmhpC/ade operon. To test this we deleted the entire genomic region containing tfmR, mhpC and ade and assessed whether the deletion of mhpC and ade restores the virulence phenotype of Xcc∆tfmR. Deletion of mhpC/ade enabled the bacteria to grow slightly better in sweet orange compared to Xcc∆tfmR but did not complement the virulence reduction phenotype of Xcc∆tfmR and leaves inoculated with the triple mutant displayed slightly weaker disease symptoms than Xcc∆tfmR (Fig. S5A and B). Additionally, we overexpressed mhpC/ade through a plasmid under control of the lac promoter in the Xcc wild type. Overexpression of mhpC/ade did not significantly alter the virulence of Xcc (Fig. S5C and D).

Fatty acids mediate the repression of the mhpC/fadE operon by TfmR

Several TFRs were reported to directly regulate fatty acid catabolism in response to long-chain fatty acids in different bacteria (Fujita et al., 2007; Kang et al., 2008) (Agari et al., 2011). FadE and MhpC/BioH are directly controlled by TfmR in Xcc, and play an essential role in the catabolism of fatty acids (DiRusso et al., 1999; Ruslan et al., 2003) (Kadisch et al., 2017). Therefore, we hypothesized that TfmR responds to fatty acids in its regulation. We monitored the effect of multiple fatty acids on DNA binding by TfmR using EMSA (Fig. 6A). Addition of 0.02% of the short-chain fatty acids, hexanoic (C6:0) acid and octanoic acid (C8:0) did not affect DNA binding, whereas the addition of 0.02% of

Fig. 5 TfmR interacts with the promoter region of mhpC/fadE. Electrophoretic mobility shift assays (EMSA) was used to determine the binding of biotin labelled DNA by GST-TfmR. (A) 20 pmol of GST or GST-TfmR was incubated with 1 ng of biotin labelled pmhpC/fadE and the indicated fold increase of unlabelled pmhpC/fadE DNA as a competitor. The experiment was repeated three times with similar results. (B) Binding affinity assay of 1 ng of biotin labelled pmhpC/fadE with the indicated amount of GST-TfmR or GST. The experiment was repeated three times with similar results.

Fig. 6 Long-chain fatty acids modulate the DNA binding affinity of TfmR. (A, B) 20 pmol of GST-TfmR was incubated for 15 min with 1 ng of biotin labelled pmhpC/fadE and binding was determined by EMSA. (A) Samples were incubated with 0.02% of the indicated fatty acids. The experiment was repeated three times with similar results. (B) Binding of pmhpC/fadE was tested in the indicated concentrations of oleic acid. The experiment was repeated four times with similar results. (C) GUS activity was determined for Xcc wild type (WT) and Xcc∆tfmR harbouring the pmhpC/fadE GUS promoter fusion. Bacteria were grown for 12 h in NB media or 24 h in XVM2 media supplemented with the indicated fatty acids. Values are means ± SE of three independent biological repeats within one experiment. The experiments were repeated three times with similar results. Asterisks indicate a significant difference (Student’s t-test, P-value < 0.05) relative to Xcc grown in NB media, which was not supplemented with fatty acids.
multiple long-chain fatty acids (i.e., myristic acid [C14:0], vaccenic acid [C18:1 cis-7], oleic acid [C18:1 cis-9], linoleic acid [C18:2 cis-9, 12] and arachidonic acid [C20:4 cis-5, 8, 11, 14]) inhibited the binding of TfmR to its target promoter (Fig. 6A). We also demonstrated that the inhibition by oleic acid is concentration dependent (Fig. 6B).

The effect of fatty acids on the promoter activity of *mhpC/fadE* was tested in Xcc wild type and XccΔtfmR in nutrient broth (NB) and XVM2 media. Addition of 0.1% myristic acid, oleic acid and vaccenic acid to Xcc culture media significantly (P-value < 0.05) induced the *mhpC/fadE* promoter in Xcc wild type by 5- to 30-fold in NB and by 3- to 5-fold in XVM2 (Fig. 6C). Exogenous supplementation of same fatty acids to XccΔtfmR cultures grown in either NB or XVM2 media did not affect the expression of the *mhpC/fadE* promoter (Fig. 6C), indicating that long-chain fatty acids positively regulate the *mhpC/fadE* promoter probably by nullifying the repressing activity of TfmR.

**TfmR is important for fatty acid metabolism**

The long-chain fatty acid dependent regulation of *fadE* and *mhpC* by TfmR led us to speculate that deletion of this regulator might alter the fatty acid metabolic profile and regulation in Xcc. First, we examined if XccΔtfmR utilizes fatty acid and non-fatty acid based carbon sources differently from the wild type. To this end Xcc wild type, XccΔtfmR and XccΔtfmR + tfmR were monitored for growth in XVM media (identical to XVM2 media minus fructose and sucrose) containing either 10 mM sucrose or 0.1% oleic acid. XccΔtfmR displayed growth retardation in XVM media supplemented with sucrose compared to the wild type and complemented strain but displayed similar growth kinetics to the wild type when the media were supplemented with oleic acid (Fig. 6A and B). XccΔtfmR also display reduced growth in XVM2 media in a similar manner to XVM + Sucrose (data not shown). Interestingly, the complemented strain, which expresses TfmR under the constitutive lac promoter, grew much slower than the wild type or the mutant strain in the presence of oleic acid (Fig. 7B). We further inspected how XccΔtfmR grows in rich media with or without exogenous supplementation of fatty acids. Bacteria were monitored for growth in NB, an undefined rich media or NB supplemented with 0.1% oleic acid. The growth rate of XccΔtfmR and XccΔtfmR complemented strain was slightly reduced compared to Xcc wild type in NB (Fig. S6A). In addition, XccΔtfmR cultures entered stationary phase upon reaching a lower population level than the wild type (Fig. S6A). The growth rate of both strains was similar to Xcc wild type when media was supplemented with oleic acid; however, XccΔtfmR entered stationary growth phase in a lower population compared to the wild type as well (Fig. S6B).

To further assess the effect of TfmR on fatty acid metabolism, we mapped key enzymes in the beta-oxidation pathways in Xcc.
(i.e. fadH [XAC1010], fadI [XAC0213], fadA [XAC2012]) and two copies of fadB (fadB1 [XAC2013], in an operon with fadA and XAC2014, and fadB2 [XAC1318]) and monitored the expression of these genes in Xcc wild type and XccΔtfmR using qRT-PCR and GUS promoter fusions in NB and XVM2 media. In a similar manner to the expression pattern of fadE, fadH exhibited significantly (P-value < 0.05) elevated mRNA transcript accumulation and promoter activity in XccΔtfmR compared to the Xcc wild type (Figs 7C and S6C). Other beta-oxidation genes showed altered expression in XccΔtfmR compared to the wild type as well: media specific activation of the fadI promoter was observed in XccΔtfmR grown in XVM2 (Fig. S6C) while the mRNA transcript of fadA was consistently elevated in XccΔtfmR grown in NB media (Fig. 7C). Direct interaction of TfmR with the promoter regions of beta-oxidation genes was determined in vitro and in vivo by EMSA and ChIP-qPCR, respectively. TfmR was able to directly bind to the fadH promoter in vitro and in vivo, whereas binding to the promoter of XAC2014/fadB1/fadA was only observed in vitro (Figs S4B, S6D and S6E). Those results suggest that TfmR directly regulates multiple components of the fatty acid catabolism pathway.

MhpC/BioH was shown to play a role in the processing of fatty methyl-esters (Kadisch et al., 2017; Ruslan et al., 2003) while FadE and FadH play an essential role in the beta-oxidation cycle (Campbell and Cronan, 2002; He et al., 1997). The constitutive expression of these enzymes can potentially cause metabolic imbalance and alter the fatty acid profile of Xcc. We determined the fatty acid profile of Xcc wild type and XccΔtfmR by gas-chromatography (GC) and found significant (P-value < 0.05) differences in the abundance of most fatty acids and in the ratio of saturated and non-saturated fatty acids as a whole. The percentage of saturated fatty acids within total fatty acids was higher in Xcc wild type compared to XccΔtfmR, while the percentage of non-saturated fatty acids was higher in XccΔtfmR compared to Xcc wild type (Table S2, and Fig. 7D). Interestingly, the major differences between the two strains within the unsaturated fatty acid (UFA) were in the branched UFAs (BUFAs): the percentage of non-branched UFA was similar in the wild type and the mutant, the percentage of iso BUFAs was significantly higher in the mutant, and the percentage of anteiso BUFAs was slightly higher in the wild type (Table S2, and Fig. 7D). These data shows that TfmR has a significant effect on the fatty acid abundance, potentially due to its effect on the expression of fatty acid catabolism genes.

**Long-chain fatty acids suppress T3SS gene expression**

We demonstrated that TfmR functions as a suppressor of fatty acid catabolism genes in the absence of long-chain fatty acids; therefore we hypothesized that fatty acid sensing or...
metabolism plays a role in T3SS gene expression. We tested the induction of the *phrpX::GUS, phrpF::GUS* and *xopAU::GUS* in Xcc wild type and *XccΔtfmR* incubated in XVM2 media supplemented with 0.1% of myristic acid, oleic acid and vaccenic acid. The addition of oleic acid and vaccenic acid significantly (P-value < 0.05) reduced the expression of the three reporters in Xcc wild type when compared to XVM2 media without fatty acids (Fig. 8A). The induction of the three reporters was not altered in *XccΔtfmR* by the addition of fatty acids (Fig. 8A). We further tested the effect of oleic acid on T3SS gene expression by quantification of T3SS and effector genes (e.g. *hrpG, hrpX, hprF, xopAU, xopN* and *xopK*) using qRT-PCR. In the wild-type *Xcc*, expression of the majority of the marker genes was reduced in XVM2 supplemented with 0.1% oleic acid compared to XVM2 (Fig. 8B). We did not observe a similar effect in *XccΔtfmR* (Fig. 8B).

The effect of oleic acid on the virulence of Xcc was also examined. We first attempted to add oleic acid to the inoculation medium and directly infiltrated the bacteria with oleic acid into sweet orange leaves, but we failed to observe any difference in virulence. We then examined the effect of oleic acid on the bacteria by pre-incubating them with XVM2 or XVM2 with 0.1% oleic acid for 24 h prior to leaf infiltration. Xcc that were pre-incubated with oleic acid displayed consistent retardation in growth compared to the control incubation in Xcc wild type but not in *XccΔtfmR* (Fig. 8C). These results indicate that fatty acids or derivatives of fatty acid metabolism play an essential role in the regulation of the T3SS and virulence in Xcc and suggest that TfmR acts as a fatty acid sensor that modulates this response.

**DISCUSSION**

In this study we identified the TetR-family transcriptional repressor TfmR as a long-chain fatty acid sensor and virulence regulator in *Xanthomonas*. We showed that deletion of *tfmR* in Xcc resulted in reduction of type III secretion and effector gene expression and subsequently attenuated the pathogenicity of Xcc *in planta*. Further analysis discovered that TfmR directly represses the expression of fatty acid metabolism associated genes and that its DNA binding ability is disrupted by long-chain fatty acids. In turn, we demonstrated that long-chain fatty acids attenuate the expression of type III secretion and effector gene in wild-type Xcc but not in the *tfmR* mutant; revealing a novel link between fatty acid sensing and virulence regulation in Xcc.

In most pathogenic bacteria virulence gene expression is under multilayer metabolic control mediated by numerous inputs (Olive and Sassetti, 2016; Rohmer et al., 2011). The HrpG/HrpX virulence regulon is well characterized in *Xanthomonas* but the specific environmental cues that control this regulon are not well understood. In all *Xanthomonas* spp. the *hrpG/hrpX* regulon is suppressed in complex rich media and induced in an optimized defined media (Astua-Monge *et al.*, 2005; Jiang *et al.*, 2013; Schulte, 1992; Tsuge *et al.*, 2002). This effect suggests that metabolic regulation of this regulon is likely to be partially under negative control by specific metabolites. In support of this hypothesis, we identified that supplementing long-chain fatty acids to the *Xcc* T3SS inducing media, XVM2, suppressed the expression of *hrpG, hrpX* and their downstream T3SS genes. In the plant apoplast simple carbohydrates are available for the bacteria to utilize as a carbon source (Lowell *et al.*, 1989) (Fatima and Senthil-Kumar, 2015; Naseem *et al.*, 2017) while long-chain fatty acids are usually associated with non-specific lipid transfer proteins (nsLTPs) making them less accessible (Liu *et al.*, 2015). On the other hand, plant leaf and fruit surface areas are enriched in hydroxylated C16 and C18 fatty acids that compose the cutin polymer, the main structural component of the plant cuticle (Lara *et al.*, 2015). Previous study of temporal and spatial gene expression in the phyllosphere has shown that the expression of *hrpG* is repressed when bacteria are localized on the leaf surface and induced once the bacteria reaches the stomata area and enter the plant apoplast (Zhang *et al.*, 2009). Therefore the negative effect of long-chain fatty acids on HrpG/HrpX virulence regulon might function as a form of metabolic environmental sensing by distinguishing the apoplast from the outer leaf surface.

Fatty acids play an essential role in the membrane, energy metabolism and signalling and their biosynthesis and degradation are subjected to tight regulation (Fujita *et al.*, 2007). The regulation of Fad in Enterobacteria is mediated by the global GntR-family transcription regulator FadR (Fujita *et al.*, 2007; Simons *et al.*, 1980). While the beta-oxidation pathway is conserved in *Xanthomonas*, it does not encode a homolog of FadR. TfmR binds to its target promoters and is released from them in the presence of long-chain fatty acid ligand. We found that this regulator represses the expression of *fadE* and *fadH* genes while not affecting the expression of the downstream beta-oxidation coding *fadB* and *fadA* genes in the experimental conditions that were tested. FadE catalyzes the production of enoyl-CoA from acyl-CoA and FadH functions as an accessory enzyme that recues 2, 4-dienoyl-CoA to enoyl-CoA when the initial Fad substrate is an UFA with double bonds (Campbell and Cronan, 2002; He *et al.*, 1997). We speculate that TfmR functions as a regulator of enoyl-CoA production while other compartments of the beta-oxidation pathway are regulated separately. Interestingly, the *Xanthomonas* fadB and *fadA* are encoded in an operon along with a TetR-family gene coding gene (XAC2014), which shares homology (36% identity, 54% positive) to *Pseudomonas aeruginosa* PsIA. PsIA was implicated to regulate the fadB/fadA operon in *Pseudomonas* (Kang *et al.*, 2008); suggesting that in *Xanthomonas* the beta-oxidation pathway is controlled at least by two independent regulators.
TfmR might contribute to virulence by affecting multiple independent pathways. While we show that expression of T3SS and effector marker genes is reduced in Xcc∆tfmR, the overexpression of HrpG only partially salvages the virulence of Xcc∆tfmR indicating that other factors contribute to the reduction in virulence. Indeed, Xcc∆tfmR displays multiple phenotypes that can affect in planta fitness independent of the T3SS. For example, reduced production of EPS and altered membrane fatty acid composition will potentially make the bacteria more sensitive to plant antimicrobial compounds (Katzen et al., 1998; Li and Wang, 2011a; Ramos et al., 2002). Furthermore, inability to utilize sucrose efficiently as a carbon source will hinder the bacterial growth during infection since sucrose is abundant in the plant and is speculated to serve as one of the main carbon sources utilized by the bacteria (Fatima and Senthil-Kumar, 2015).

The mechanism by which TfmR and long-chain fatty acids regulate the hrpG/hrpX regulon remains elusive. Direct transcriptional control of the regulon by TfmR is unlikely since TfmR does not bind to the hrpG and hrpX promoter regions. We speculated that the hrpG/hrpX regulon might be indirectly affected by the constitutive expression of mhpC, fadE or fadH. However, we disproved this hypothesis in this study. The HrpG and HrpX were found to be directly controlled in multiple ways and several regulators were identified to affect these proteins in different levels (Andrade et al., 2014; Li et al., 2014; Lu et al., 2011; Zhou et al., 2018); transcriptional control of one or several of these regulators by TfmR is possible. Alternatively, the effect of TfmR and long-chain fatty acids on the hrpG/hrpX regulon might be a consequence of altered cell metabolic state. Another mechanism that might play a role in the function of TfmR is production and response to DSF (Zhou et al., 2017a). DSF is a medium-chain fatty acid derivative and its production might be affected by the effect of TfmR on fatty acid metabolism and can also potentially directly affect the ability of TfmR to bind to its target promoters by acting as a fatty acid ligand. To test this hypothesis we monitored the expression of in rpfB, rpfF, rpfC and rpfG in Xcc∆tfmR but did not observe any differential gene expression compared to the wild type (Fig. S7). We also tested whether TfmR can bind to the rpfB/rpfF promoter in vivo but did not observe any significant interaction (Fig. S3B). Identification of novel targets of TfmR and further examination of DSF production and sensing by Xcc∆tfmR will provide new insights into the effect of TfmR on pathogenicity and cell physiology.

While further study should be conducted regarding the link between TfmR and the control of the hrpG/hrpX regulon our observation leads to a proposed model (Fig. 9): When bacteria are found in environments that contain low abundance of long-chain fatty acids, such as the plant apoplast, TfmR binds to the promoter region of mhpC, fadE and fadH to suppress their expression and indirectly promote the expression of the hrpG/hrpX regulon. In a long-chain fatty acid enriched environment, such as the plant leaf surface, long-chain fatty acids they act a ligand and bind to TfmR thus, releasing it from its target genes and induce the expression of MhpC, FadE and FadH, which allows the utilization of fatty acids as an energy source by introducing them into the beta-oxidation pathway and produce acetyl-coA. In parallel, binding of long chain-fatty acids by TfmR suppress the expression of the hrpG/hrpX regulon through an unknown mechanism. By catabolizing the fatty acids through the beta-oxidation pathway the level of long-chain fatty acids is reduced, freeing TfmR and allowing it to again bind to and suppress the expression of its target genes and keeping lipid homeostasis.

**EXPERIMENTAL PROCEDURES**

**Bacterial strains, plasmids and primers**

The bacterial strains and plasmids used in this study are listed in Table S3. Oligonucleotides used in this study are listed in Table S4. *Xanthomonas citri* was grown at 28 °C in NB medium (Beef extract 3 g/L, Peptone 5 g/L), nutrient agar (NA) plates, XVM2 media (Wengelnik and Bonas, 1996) or modified XVM2 media (XVM) in which fructose and sucrose were replaced with 0.1% oleic acid. *E. coli* was grown at 28 °C in Luria-Bertani (LB) medium at 37 °C. When required, growth media was supplemented...
with gentamicin (5 \(\mu\)g/mL), kanamycin (50 \(\mu\)g/mL), ampicillin (100 \(\mu\)g/mL) and spectinomycin (200 \(\mu\)g/mL).

**Tn5 transposon screen**

Xcc 306 bacteria containing pGUS:phpF reporter were transformed with Tn5 transposon using EZ-Tn5™ <KAN-2>Tnp Transposome™ Kit (Lucigen Corporation, Middleton, WI, USA) and plated on XVM2 agar (1.5%) supplemented with X-gluc (0.01 mg/mL), gentamicin and kanamycin yielding ~10,000 colonies. Five hundred colonies, which displayed reduced or enhanced intensity of blue compared to neighbouring colonies were picked into new XVM2/X-gluc plates in parallel to Xcc 306 + pGUS:phpF. Two hundred and forty colonies that displayed consistent difference in GUS activity from Xcc 306 were used for plant inoculation assays on Valencia sweet orange and symptoms were scored visually 5 days after inoculation. The transposon integration site was determined in 16 clones, which displayed reduced virulence compared to Xcc 306 in three independent experimental repeats using touchdown PCR (Levano-Garcia et al., 2005).

**Production of Xcc deletion and complemented strains**

To produce deletion mutants in tfmR, mhpC and fadE, 740 bp to 900 bp fragments (according to primers listed in Table S4) upstream and downstream of the ORF were amplified with genomic DNA of Xcc 306. The two amplicons were combined together into one fragment using overlap PCR and cloned into the pOK1 suicide vector (Huguet et al., 1998). The deletion vector was transformed into Xcc by electroporation and markerless deletion mutants were produced using a two-step sucrose counter-selection procedure (Zhou et al., 2015). For complementation of XccΔtfmR, TfmR (XAC3052) or hrpG (XAC1265) were amplified from genomic DNA of Xcc 306, cloned into pBBR1MCS-5 and introduced into Xcc wild type.

**Motility, EPS production, extracellular enzymes and catalase activity and stress resistance assays**

Motility and production of exopolysaccharides (EPS) were quantified as previously described (Li and Wang, 2011b). For analysis of protease, amylase and cellulase activity bacteria (10^8 CFU/mL) were spotted on NA plates containing 1% skimmed milk, 1% carboxymethyl cellulose or 0.5% starch and extracellular activity was quantified as described previously (Szczenzy et al., 2010; Zhou et al., 2017b). Catalase activity assay was conducted as described by (Zhou et al., 2017b); \(H_2O_2\) degradation was quantified using Pierce Quantitative Peroxide Assay Kit (Thermo Fisher scientific, Waltham, MA, USA). Resistance to SDS and \(H_2O_2\) were conducted as described by (Li and Wang, 2012).

**Pathogenicity and HR assays**

For pathogenicity assays expanded leaves of 2-year-old Valencia sweet orange were infiltrated with bacterial suspensions (10^6 CFU/mL for monitoring bacterial growth; 10^8 CFU/mL for monitoring symptoms development) in 10 mM MgCl₂ using a needless syringe and plants were kept in a greenhouse at 28 °C under natural light conditions. Bacterial growth was quantified as previously described (Teper et al., 2018).

For monitoring HR-like cell death and quantification of ion leakage 4-week-old *N. benthamiana* plants were infiltrated with 5 x 10^8 CFU/mL Xcc bacteria in 10 mM MgCl₂ using a needless syringe. Ion leakage was measured as described (Teper et al., 2018) using CON 700 benchtop conductivity meter (OAKTON Instruments, Vernon Hills, IL, USA).

**GUS assays**

To generate GUS reporter plasmid (pGUS) *E. coli* GPI-glucuronidase (gus) gene followed by a T7 terminator were cloned in reverse orientation to the lac promoter in pBRR1MCS-5. To generate GUS promoter fusions constructs the putative promoter regions of the tested genes (elaborated in Table S3) was cloned upstream to the gus gene in pGUS.

GUS assay was conducted as described (Jiang et al., 2009). GUS activity was quantified by arbitrary units (AU) and determined as A405 / (time in min × total protein in \(\mu\)g × 0.02).

**RNA isolation and qRT-PCR**

RNA was isolated from bacteria and plant tissue using TRizol™ Reagent (Invitrogen, Carlsbad, CA, USA) according to provided instructions. RNA samples (1 \(\mu\)g) were reverse-transcribed using qScript cDNA Synthesis Kit (Quanta BioSciences, Inc. Gaithersburg MD, USA) and subjected to qRT-PCR using gene specific primers (Table S4). cDNAs were amplified using the SYBR Premix Ex Taq II (Clontech Laboratories, Inc. Mountain View, CA, USA) and subjected to qRT-PCR using gene specific primers (Table S4). cDNAs were amplified using the SYBR Premix Ex Taq II (Clontech Laboratories, Inc. Mountain View, CA, USA) and subjected to qRT-PCR using gene specific primers (Table S4).

**Protein purification and electrophoretic mobility shift assay**

TfmR was cloned into the pGEX-4T-1 GST fusion expression vector and transformed into *E. coli* Rosetta strain. GST-TfmR was purified as previously described (Teper et al., 2018).
Probe oligonucleotides were amplified from genomic DNA of Xcc 306 using 5′- Biotin-TEG modified primers (IDT Inc, Coralville, IA, USA) (Table S4). EMSA was conducted using the LightShift Chemiluminescent EMSA Kit (Thermo Fisher, Waltham, MA, USA) according to manufacturer’s instructions. For determination of DNA binding affinity band intensity was quantified using ImageJ (https://imagej.nih.gov/ij/) and KD value was calculated as previously described (Heffler et al., 2012).

Chromatin Immunoprecipitation-Quantitative Polymerase Chain Reaction (ChIP-qPCR)

ChIP assays were done as described by (Pandey et al., 2016) with modifications. Briefly, 100 mL of XccΔtfmR contacting pBBR1MCS-5 (empty vector control) or pBBR1MCS-5:tfmR-HA were grown for 36 h in NB media. Cells were pelleted, and resuspended in Tris-buffered saline (TBS) and cross-linked with 0.75% formaldehyde for 30 min. Fixed cells were washed with TBS and sonicated until DNA was broken into 500 bp to 1000 bp fragments. For immunoprecipitation, cell lysate was incubated with Monoclonal Anti-HA−Agarose (Sigma-Aldrich, St. Louis, MO, USA) as and IP was performed as instructed by the manufacturer. Protein-DNA complex was eluted as described (Pandey et al., 2016). Relative representation of potential target promoter sequences was quantified using qPCR (primers listed in Table S4) samples were normalized by using primers directed to the gyrA promoter, which was found to not interact with TfmR in vitro.

Fatty acid methyl ester analysis

Three-day old cultures of Xcc wild type and XccΔtfmR were recultured on three NA plates and incubated at 28 °C for 48 h and 50 mg of bacteria were scooped from each NA plate and frozen in liquid nitrogen. Samples were saponificated, methylated and analysed for fatty acid content using gas chromatography by Microbial ID, Inc. (Newark, DE, USA).

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WENGENLNIK, K. AND BONAS, U. (1996) HRPXV, AN ARA C-TYPE REGULATOR, ACTIVATES MUTANTS. (A) BACTERIAL CULTURES OF XCC WILD TYPE AND THE INDICATED Tn5 XCC MUTANTS CARrying THE pHRP-F:GUS REPORTER PLASMID USING PNPG AS A SUBSTRATE. GUS ACTIVITY VALUES WERE STANDARDIZED TO A VALUE REPRESENTING RELATIVE ACTIVITY TO XCC WILD TYPE. VALUES ARE MEANS ± SE OF SIX INDEPENDENT BIOLOGICAL REPEATS TAKEN FROM THREE SEPARATE EXPERIMENTS (EACH CONTAINING TWO REPEATS). ASTERISKS INDICATE A SIGNIFICANT DIFFERENCE (STUDENT’S T-TEST, P-VALUE < 0.05) RELATIVE TO XCC WILD TYPE. (C) SWEET ORANGE LEAVES WERE SYRINGE-INFILTRATED WITH SUSPENSIONS (1 × 10⁸ CFU) OF XCC WILD TYPE (WT) AND THE INDICATED Tn5 XCC MUTANTS. PLANTS WERE PHOTOGRAPHED 5 DAYS LATER. THE EXPERIMENTS WERE REPEATED AT LEAST THREE TIMES WITH SIMILAR RESULTS.

Fig. S2 CHARACTERISTICS OF TMFR. (A) PHYSICAL MAP OF THE GENOMIC REGION SURROUNDING XAC3052 TFMFR SHOWING THE TRANSPOSITION INSERTION SITES OF BOTH Tn5 MUTANTS, THE FRAGMENT DELETED IN XCCΔTFMR (BETWEEN THE TWO RED DOTTED LINES) AND THE LOCATION OF THE VALIDATION PRIMERS USED IN D (TWO BLACK ARROWS). (B) SWEET ORANGE LEAVES WERE SYRINGE-INFILTRATED WITH SUSPENSIONS (1 × 10⁸ CFU ML) OF XCC WILD TYPE (WT) AND TWO TRANSPONSON MUTANTS IN TFMFR (XCC TFMFRTn5-1 AND XCC TFMFRTn5-2). PHOTOGRAPH WAS TAKEN AT 5 DAYS POST-INOCULATION. THE EXPERIMENT WAS REPEATED THREE TIMES WITH SIMILAR RESULTS. (C) PROTEIN ALIGNMENT OF E. COLI TetR (PU17_12890) AND HOMOLOGS OF TMFR IN Xanthomonadales USING CLUSTAL OMEGA MULTIPLE SEQUENCE ALIGNMENT TOOL UNDER DEFAULT SETTING (HTTPS://WWW.EBI.EC.UK/TOOOLS/MSA/CLUSTALO). THE REPRESENTATIVE TMFR HOMOLOGS WERE TAKEN FROM THE GENOMES OF Xanthomonas citri subsp. citri (XCC, XAC3052), Xanthomonas albinelines GPE PC73R (Xalb, XALC_2196), Stenotrophomonas maltophilia K279a (Smilt, Smil3646), Pseudoxanthomonas spadix BD-a59 (Pxs, DSC_05240), Lysobacter capsica 55 (Lbc, LC55x_1220) AND Luteimonas sp. JM171 (Lum, BG9P97_07680). GREEN MARKED AMINO ACIDS INDICATE THE AMINO ACID IS COMPLETELY CONSERVED IN ALL Xanthomonadales TMFR HOMOLOGS, YELLOW MARKED AMINO ACIDS INDICATE THE AMINO ACIDS ARE CONSERVED IN ALL Xanthomonadales XAC3052 HOMOLOGS AND E. coli TetR. (D) DELETION OF XAC3052 WAS VALIDATED BY PCR USING THE PRIMERS INDICATED BY THE BLACK ARROWS IN ‘A’.

Fig. S3 CHARACTERIZATION OF XCCΔTFMR. (A) MOBILITY ASSAY. BACTERIAL SUSPENSIONS (1 × 10⁸ CFU/mL) OF THE INDICATED STRAINS WERE SPOTTED (5 µL) ON SOFT NA PLATES (0.4%). COLONY DIAMETER WAS QUANTIFIED 3 DAYS LATER. VALUES ARE MEANS ± SE OF THREE INDEPENDENT BIOLOGICAL REPEATS WITHIN ONE EXPERIMENT. THE EXPERIMENTS WERE REPEATED THREE TIMES WITH SIMILAR RESULTS. ASTERISKS INDICATE A SIGNIFICANT DIFFERENCE (STUDENT’S T-TEST, P-VALUE < 0.05) RELATIVE TO XCC WILD TYPE. (B, C) EXTRACELLULAR HYDROLYSIS ACTIVITY OF XCCΔTFMR. SUSPENSIONS (1 × 10⁸ CFU/mL) OF XCC WILD TYPE (WT), XCCΔTFMR AND XCCΔTFMR + TFMFR WERE SPOTTED (10 µL) ON NA PLATES CONTAINING EIGHT PERCENT CARBOXYMETHYLCELLULOSE (CMC), 1% SKIMMED MILK AND 0.5% STARCH. PLATES WERE PHOTOGRAPHED AND HALO DIAMETER WAS QUANTIFIED (AFTER INCUBATION PERIOD OF 72 HRS). VALUES ARE MEANS ± SE OF THREE INDEPENDENT BIOLOGICAL REPEATS WITHIN ONE EXPERIMENT. THE EXPERIMENTS WERE REPEATED TWO TIMES (FOR STARCH) OR THREE TIMES (FOR CMC AND SKIMMED MILK) WITH SIMILAR RESULTS. ASTERISKS INDICATE A SIGNIFICANT DIFFERENCE (STUDENT’S T-TEST, P-VALUE < 0.05) RELATIVE TO XCC WILD TYPE. (D) CATALASE ACTIVITY WAS TESTED IN THE CELL EXTRACTS OF THE INDICATED STRAINS. REPRESENTED VALUES WERE STANDARDIZED FOR CATALASE ACTIVITY OF XCC WILD TYPE (SET AS 100%). VALUES ARE MEANS ± SE OF EIGHT INDEPENDENT BIOLOGICAL REPEATS TAKEN FROM THREE SEPARATE EXPERIMENTS (EACH

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containing two or three repeats). (E) The indicated strains were challenged with 0.03% H₂O₂ or 0.1% SDS. Resistance to H₂O₂ and SDS was quantified as the relative survival rate of XccΔtfmR and XccΔtfmR + tfmR compared to wild type (set as 100). Values are means ± SE of three independent biological repeats within one experiment. The experiments were repeated twice with similar results.

Fig. S4 Direct interaction between TftR and putative target promoters. (A) EMSA was used to determine the binding of biotin labelled DNA by GST-TfmR in vitro. 35 pmol of GST or 20 pmol of GST-TfmR was incubated with 2 ng of the indicated DNA fragments. The experiment was repeated four times with similar results. (B) ChIP-qPCR was used to determine in vivo interaction between TftR and the indicated promoters. Fixed extracts from the XccΔtfmR + tfmR-HA or XccΔtfmR + empty vector (EV) were fragmented and the immunoprecipitated (IP) using HA agarose. Representation of the promoter sequences was standardized to the relative representation of the gyrA promoter. Values represent the average relative representation of the indicated promoter sequences in six independent (conducted in three separated experiments) TfmR HA IP samples compared to EV control after logarithmic transformation. Asterisks indicate a significant difference (Student’s t-test, P-value < 0.05) compared to the EV.

Fig. S5 The contribution of TfmR to virulence is not dependent on mhpC fadE. Sweet orange leaves were syringe-infiltrated with suspensions (1 × 10⁸ CFU/mL for A and C, 1 × 10⁶ CFU/mL for B and D) of the indicated Xcc strains. (A, C) pictures were taken 5 days post-inoculation. The experiments were repeated three times with similar results. (B, D) In planta bacterial population was determined at the indicated days post inoculation. Values are means ± SE of at least three independent biological repeats within one experiment. The experiments were repeated twice with similar results. Letters denote significant differences based on analysis of variance (ANOVA) and comparisons for all pairs using Student’s t-test (P-value < 0.05).

Fig. S6 Regulation of beta oxidation pathway genes by TfmR. (A, B) Growth of Xcc wild type (WT), XccΔtfmR, XccΔtfmR + tfmR in NB media (A) or NB media containing 0.1% oleic acid (B). Values are means ± SE of three independent biological repeats within one experiment. The experiments were repeated three times with similar results. (C) GIS activity in bacterial cultures grown in NB or XVM2 media was determined for Xcc wild type (WT) and XccΔtfmR harbouring the indicated GUS promoter fusions. Values are means ± SE of three independent biological repeats within one experiment. The experiments were repeated three times with similar results. Asterisks indicate a significant difference (Student’s t-test, P-value < 0.05) relative to Xcc wild type. (D) EMSA was used to determine the binding of 1 ng of biotin labelled DNA representing the indicated promoters by 35 nmol of GST or 20 pmol of GST-TfmR. The experiment was repeated twice with similar results. (E) Inhibition or promoter binding by TfmR by addition of 100 ng competitor DNA (X100 competitor). The experiment was repeated twice with similar results.

Fig. S7 mRNA abundance of the indicated genes was quantified by qRT-PCR in bacterial cultures after incubation in XVM2 media for 24 h. gyrA mRNA abundance was used for normalization. Values are means ± SE of three independent biological repeats within one experiment. The experiments were repeated at least three times with similar results. No significant difference was observed (Student’s t-test, P-value < 0.05) between the Xcc wild type (WT) and XccΔtfmR.

Table S1 Virulence deficient Tn5 mutants identified in this study.
Table S2 Fatty acid abundance (%) in Xcc wild type and XccΔtfmR.
Table S3 Bacterial strains and plasmids used in this study.
Table S4 Primers used during this study.