XagR, a LuxR Homolog, Contributes to the Virulence of *Xanthomonas axonopodis* pv. *glycines* to Soybean

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A novel *luxR* homolog, termed *XagR*, in *Xanthomonas axonopodis* pv. *glycines*, the cause of soybean pustule, controls expression of *pip*, *yapH*, and at least 77 other genes. Although XagR and Pip are required for full virulence of *X. axonopodis* pv. *glycines* to soybean, constitutive overproduction of XagR suppresses infection. The *xagR*-dependent induction of *pip* occurs in planta only 2 days or more after inoculation. Although the transcription of *xagR* appears constitutive, XagR accumulates only in cells that have colonized soybean plants for more than 2 days suggesting that some components produced during the infection process mediate post-transcriptional control, likely by protecting XagR from proteolytic degradation. XagR modulates the adhesiveness of the pathogen during the infection process by suppressing the adhesin YapH. Although *yapH* mutants incite more infections of soybean leaves than the wild-type strain when topically applied under dry conditions, the mutant causes fewer infections when leaves are subject to simulated rain events after inoculation. Likewise, *yapH* mutants and cells in which XagR was overexpressed exhibited much more egress from infected leaves than the wild-type strain. Thus, XagR differentially modulates expression of a variety of genes during the infection process in response to feedback from plant molecules elaborated during infection to coordinate processes such as invasion, infection, and cell egress needed to complete the disease cycle.

As plant-pathogenic bacteria engage in intimate interactions with host plants, there is growing evidence that they exploit chemical cues provided by the plant in order to orchestrate sequential expression of traits involved in the interaction. For example, phenolic glycosides and sugars are used as cues for the production of phytotoxins by different pathovars of *Pseudomonas syringae* (Mo and Gross 1991; Mo et al. 1995). The expression of genes involved in type III secretion of effectors from many bacterial species into plants is likewise dependent on the presence of various plant-derived molecules as well as a low osmolarity environment and pH values typical of the plant apoplast (Arlat et al. 1992; Huynh et al. 1989; Rahme et al. 1992; Schulte and Bonas 1992; Wei et al. 1992; Xiao et al. 1992). Unknown compounds present in plant extracts also modulate extracellular enzyme production in soft-rotting plant pathogens (Hugouvieux-Cotte-Pattat et al. 1992, 1996). There

is a rich literature that demonstrates that the initial interactions of many rhizobia and *Agrobacterium tumefaciens* with host plants is dependent on recognition of specific plant compounds such as flavonoids and other phenolics (Bergman et al. 1988; Caetano-Anolles et al. 1988; Hawes and Smith 1989; Parke et al. 1987).

In addition to such extrinsic factors, many bacteria also coordinate gene expression in response to changes in intrinsic factors, often used to indicate local cell density in a process referred to as quorum sensing (QS). In most gram-negative bacteria, the QS system is dependent on the production and perception of N-acyl homoserine lactones by two proteins belonging to the LuxI-LuxR families (Fuqua and Greenberg 2002). Although the signal is generated by an N-acyl homoserine lactone synthase in the LuxI family of proteins, it is perceived by specific transcriptional regulators (LuxR homologs). LuxR homologs contain an AHL-binding region, which induces multimerization while a helix-turn-helix domain is involved in activation of transcription of target genes (Choi and Greenberg 1991; Poellinger et al. 1995; Stock et al. 1990; Stevens and Greenberg 1997; Stevens et al. 1994).

Numerous bacterial plant pathogens have been reported to harbor so-called “orphan” LuxR homologs without an accompanying LuxI homolog, suggesting that they are not involved in classical cell density-dependent gene regulation. These LuxR homologs commonly regulate a variety of genes or functions involved in pathogenicity. For example, AviR and AvhR, LuxR homologs of *A. vitis*, are associated with induction of the hypersensitive response in tobacco as well as necrosis in grape (Hao et al. 2005). LuxR homolog CepR2 positively regulates pyochelin production in *Burkholderia cenocepacia* by controlling transcription of one of the operons required for its synthesis (Malott et al. 2009). Likewise, ExpR activates an RNA-binding protein in *Erwinia carotovora* subsp. *carotovora* that functions as a negative regulator of extracellular enzymes and proteins as well as secondary metabolites (Cui et al. 2005).

Curiously, recent work has shown that LuxR homologs in some plant-associated bacteria are responsive to plant signal molecules. PsoR, a LuxR homolog in the plant-growth-promoting rhizobacterium *P. fluorescens*, is protected from proteolytic degradation in the presence of macerated rice and wheat and regulates expression of target genes that contribute to its biocontrol activity (Subramoni et al. 2011). Interestingly, LuxR homologs are required for full virulence of the vascular pathogens *Xanthomonas campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*. The abundance of XccR, a LuxR homolog in *X. campestris* pv. *campestris*, was enhanced in the presence of the host plant cabbage and upregulated the neighboring gene encoding proline iminopeptidase (*pip*); both *xccR* and *pip* were required for full virulence of *X. campestris* pv. *campestris* to

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cabbage (Zhang et al. 2007). Similarly, the abundance of OryR that induces *pip* in *X. oryzae* pv. *oryzae* is elevated in the presence of a compound present in both healthy and infected rice. Moreover, *chsA* encoding 1,4- β -cellobiosidase is positively regulated by OryR but the *oryR* promoter itself is negatively autoregulated by OryR and its autoregulation is independent of the rice signal molecule. OryR also plays a role in *X. oryzae* pv. *oryzae* virulence to rice because an OryR mutant incites less disease (Ferluga and Venturi 2009).

Bacterial pustule, caused by *X. axonopodis* pv. *glycines*, is one of the most serious diseases of soybean in several parts of the world. The occurrence of disease is strongly favored by high temperatures ($\geq 30^{\circ}\text{C}$) and humidity ($>60\%$ relative humidity) (Prathuangwong and Amnuaykit 1987). Erumpent pustules, presumably favoring bacterial egress, are surrounded by yellow halos. Severe disease results in premature defoliation and decreasing yield and seed quality (Morgan 1963; Weber et al. 1966). Relatively little is known of the infection process in *X. axonopodis* pv. *glycines*. Although *X. axonopodis* pv. *glycines* produces indole acetic acid and cytokinin, both of which presumably contribute to pustule formation (Fett and Dunn 1987; Millar 1955), the role of an unidentified toxin (Hokawat and Rudolph 1993), cellulases and protopectinases (Hokawat and Rudolph 1993), and bacteriocins (Fett et al. 1987) in the infection process of soybean are less well established.

X. axonopodis pv. *glycines* has a very different lifestyle than the better-studied vascular pathogens *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* because it infects through stomata and wounds on soybean leaves and causes hypertrophy of host cells. After invasion into the leaf through stomata, bacteria multiply within the substomatal chambers and intercellular spaces of the spongy mesophyll (Jones and Fett 1985). This disease process is quite distinct from that of *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*, which invade the vascular system of their host plants. Although *X. axonopodis* pv. *glycines* appears to harbor some regulators of virulence in common with *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*, such as a *luxR* homolog we term *xagR*, it seemed likely that its distinct interactions with soybean would necessitate a different role for such a regulator than that found in vascular pathogens. Thus, in this study, we investigated the role of *xagR* in interactions

with soybean and elucidated its regulon to better understand the infection process. Evidence is provided that host factors contribute to the stabilization of XagR that, in turn, provides temporal control of a variety of traits involved in movement on and attachment to plants.

RESULTS

X. axonopodis pv. *glycines* genome description and presence of *xagR* and *pip*.

The draft genome sequence of *X. axonopodis* pv. *glycines* 12-2 was determined using a combination of paired-end 454 GS-FLX pyrosequencing and paired-end Illumina/Solexa sequencing. The reads from 454 and Illumina were mapped against the finished genome sequence of *X. axonopodis* pv. *citri* 306 as a scaffold to create an *X. axonopodis* pv. *glycines* consensus sequence. Those reads that could not be mapped onto the reference strain were assembled de novo to produce contigs unique to *X. axonopodis* pv. *glycines*. The combined *X. axonopodis* pv. *glycines* consensus sequences and unmapped contigs were used to identify most of the functional genes in *X. axonopodis* pv. *glycines*. The combined Illumina and 454 platforms generated a total of 41,792,635 high-quality filtered sequence reads with an average read length of 71.4 bp. Average coverage was more than 400-fold. This yielded 489 contigs with an N50 (length-weighted median, such that 50% of the genome is contained in contigs of size N50 or greater) value of 20,859 bp. In total, 5,062 putative coding genes (open reading frames [ORF]) were identified in the *X. axonopodis* pv. *glycines* genome.

The sequenced *X. axonopodis* pv. *glycines* genome revealed the presence of a putative *luxR* homolog that we termed *xagR*. XagR is a protein of 254 amino acids with a predicted molecular mass of 29.7 kDa. Analysis of XagR with the Conserved Domain Architecture Retrieval Tool revealed that it contains an autoinducer-binding domain (pfam03472) located in residues 17 to 177 and a helix-turn-helix DNA-binding domain from residues 189 to 246, characteristic of the LuxR family of proteins (Fig. 1A). XagR shared the highest level of identity (99%) to the AhyR/AsaR family transcriptional regulator from *X. axonopodis* pv. *citri* 306, whereas it exhibited 96, 93, 92, and 86% identity to a homolog in *X. campestris* pv. *vesicatoria* 85-10 and *X. oryzae* pv. *oryzicola* BLS256, as well as OryR from *X. oryzae* pv. *oryzae* and XccR from *X. campestris* pv. *campestris*, respectively. *xagR* is located upstream of and in the same orientation as a homolog of *pip* in *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris*, with an intergenic sequence of 384 bp. Within the 384-bp intergenic region, a 20-bp palindromic sequence centered -70.5 bp from the translation start site of *pip* (Fig. 1B) was found that is highly similar to the *lux* box sequences located in a similar position in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* (Fig. 1C) (Ferluga et al. 2007; Zhang et al. 2007).

Fig. 1. A, Structure of XagR from *Xanthomonas axonopodis* pv. *glycines* with schematic representation of the positions of the AHL-binding and the helix-turn-helix-DNA binding domains typical of quorum-sensing LuxR family regulators. **B**, Analysis of the gene encoding proline iminopeptidase (*pip*) promoter locus within the 384-bp intergenic region upstream from the *pip* gene and location of a putative palindromic *lux* box sequence. **C**, Alignment of the putative *lux* boxes associated with *pip* genes in *X. axonopodis* pv. *glycines*, *X. campestris* pv. *campestris*, and *X. oryzae* pv. *oryzae*.

Table 1. Virulence of *Xanthomonas axonopodis* pv. *glycines* mutants on soybean^a

Strains	Lesions per plant
Wild type	254 ± 80
<i>xagR</i> mutant	97 ± 29
<i>pip</i> mutant	102 ± 32
<i>xagR/pip</i> double mutant	96 ± 45
XagR ⁺	297 ± 84
XagROX	86 ± 32
XagID3706 mutant	183 ± 24
XagID3707 mutant	137 ± 22
<i>yapH</i> mutant	433 ± 57

^a Data shown are the averages ± standard deviations.

xagR and *pip* contribute to virulence of *X. axonopodis* pv. *glycines* to soybean.

Disruption of *xagR* significantly reduced ($P < 0.05$) the incidence of infection of soybean leaves when cells were sprayed onto a susceptible soybean cultivar (Table 1). Because *pip* is regulated by a *luxR* homolog in both *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* and contributes to their virulence in cabbage and rice, respectively, we assessed the virulence of a *pip* mutant and an *xagR/pip* double mutant of *X. axonopodis* pv. *glycines*; both exhibited reduced virulence similar to that of the *xagR* mutant alone (Table 1). An *xagR* mutant complemented with *xagR* in trans on a low-copy number vector (*xagR*⁺) exhibited virulence to soybean similar to the wild-type *X. axonopodis* pv. *glycines* strain (Table 1). Curiously, the virulence of the *xagR*-overexpressing strain XagROX was less than that of the wild-type strain (Table 1). In order to determine whether the virulence deficiencies of the *xagR* and *pip* mutants were associated with decreased bacterial growth in planta, we monitored the growth of cells infiltrated into soybean leaves. The population sizes of the mutants were similar to that of the *X. axonopodis* pv. *glycines* wild-type strain at a given time after infiltration into soybean (Supplementary Fig. S1). It was noteworthy that the numbers of cells of *X. axonopodis* pv. *glycines* increased progressively in the plant for up to 7 days, when symptom development became apparent. Thus, the population dynamics of *X. axonopodis* pv. *glycines* is quite distinct from that seen with many other foliar pathogens such as *P. syringae*, in which bacterial multiplication in the plant typically ceases 2 or 3 days after inoculation. These results indicated that XagR and Pip are required for full virulence of *X. axonopodis* pv. *glycines* to soybean and that much of the contribution of XagR might be via any effect it might have

on expression of *pip*. Curiously, these virulence effects are apparently not via any effects on the ability of the cells to grow within the plant.

XagR is required for *pip* induction in planta.

Because the *pip* genes are expressed in a *luxR* homolog-dependent manner in both *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae*, and are induced in host plants in *X. campestris* pv. *campestris* and by rice extracts in *X. oryzae* pv. *oryzae* (Ferluga et al. 2007; Ferluga and Venturi 2009; Zhang et al. 2007), we explored the context-dependent expression of *pip* in *X. axonopodis* pv. *glycines*. The expression of *pip* was assessed using a promoterless ice nucleation (*inaZ*) reporter gene cloned downstream of the *pip* promoter-containing region in pPip-KI. The ice nucleation activity of the *X. axonopodis* pv. *glycines* wild-type strain and an *xagR* mutant carrying pPip-KI were compared when grown under different conditions, including Luria-Bertani (LB) medium, M9 minimal medium, M9 minimal medium containing macerates of either healthy or *X. axonopodis* pv. *glycines*-infected soybean, as well as in cells recovered from infiltrated soybean 2 days after inoculation. The ice nucleation activity of cells of the *X. axonopodis* pv. *glycines* wild-type strain but not an *xagR* mutant was markedly increased after inoculation into susceptible soybean. No induction of *pip* was seen in either strain when grown in media containing soybean macerates or in the resistant soybean 'Williams 82' (Fig. 2A). Thus, XagR appears to regulate the expression of at least *pip* within susceptible plants, and assessment of *pip* expression provides a convenient means by which the activity of XagR can be monitored.

Given that XagR has the characteristic signatures of an AHL-dependent response regulator, we tested whether its regulatory ability was responsive to the presence of AHL. No induction of *pip* was observed when structurally different AHL (C4, C6, C8, C10, C12, C14, oxoC6, and oxoC8) were added to the culture medium at a final concentration of 1 μ M (data not shown).

Because the accumulation of OryR and XccR in *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris*, respectively, was found to be responsive to signal molecules present in their compatible hosts, we assessed whether XagR function was as responsive to these plant species as it was in soybean. The ice

Fig. 2. A, Ice nucleation activity indicative of the gene encoding proline iminopeptidase (*pip*) promoter activity in a wild-type strain of *Xanthomonas axonopodis* pv. *glycines* (open bars) and an *xagR* mutant (solid bars) harboring a *pip:inaZ* fusion when grown in various culture media and when inoculated into soybean plants. **B,** Relative abundance of *xagR* transcripts in various strains grown in different culture media and in planta to that observed in a wild-type strain of *X. axonopodis* pv. *glycines* grown in M9 media as determined by real-time reverse-transcriptase polymerase chain reaction. Vertical bars represent the standard error of mean log ice nucleation activity or relative gene expression.

Fig. 3. Ice nucleation activity indicative of the gene encoding proline iminopeptidase (*pip*) promoter activity in a wild-type strain of *Xanthomonas axonopodis* pv. *glycines* (open bars) and an *xagR* mutant (solid bars) harboring a *pip:inaZ* fusion when recovered from rice, cabbage, soybean 'Spencer' (susceptible), soybean 'Williams 82' (resistant), and tobacco 2 days after inoculation. Vertical bars represent the standard error of mean log-transformed ice nucleation activity.

nucleation activity of wild-type *X. axonopodis* pv. *glycines* harboring a *pip:inaZ* fusion but not the *xagR* mutant was increased when infiltrated into rice, cabbage, and soybean but not into tobacco (Fig. 3). This result suggests that the putative signal molecule that interacts with XagR might be common to many plants, although it is not likely the same signal that interacts with OryR because macerates of rice but not soybean induce OryR (Ferluga and Venturi 2009).

Quantitative reverse-transcription polymerase chain reaction (RT-PCR) was performed to determine whether *pip* transcript abundance, in addition to its promoter activity, was elevated in *X. axonopodis* pv. *glycines* after inoculation into soybean. The levels of *pip* transcript in wild-type *X. axonopodis* pv. *glycines* cells recovered from infected soybean plants was 18-fold higher than that in either cells grown in culture or in an *xagR* mutant (Fig. 2B). As observed with reporter gene fusions, there was no increased accumulation of *pip* transcript in wild-type cells grown on media containing soybean macerates, and little *pip* transcript in the *xagR* mutant in most conditions. Thus, the induction of *pip* is dependent on XagR and appears to require specific molecules in living soybean plants that are either labile or not easily extracted.

Temporal expression of *pip* during an infection event.

Increased *pip* expression after inoculation of *X. axonopodis* pv. *glycines* into soybean suggested that a molecule or molecules present in soybean was responsible for altering XagR function. However, we lacked information about whether such a factor was preformed in the plant or whether it increased after plant infection, as was suggested for the factor found in *X. oryzae* pv. *oryzae*-infected rice (Ferluga and Venturi 2009; Ferluga et al. 2007). Thus, we measured the expression of *pip*

as a function of time after inoculation into soybean. The ice nucleation activity of wild-type *X. axonopodis* pv. *glycines* cells harboring a *pip:inaZ* fusion increased only slowly after inoculation into soybean, and the highest expression was observed 72 h after inoculation and then decreased slowly with time (Fig. 4). The ice nucleation activity of cells of an *xagR* mutant harboring the reporter gene remained low at all times after inoculation (Fig. 4). Considerable and similar growth of the wild-type strain and *xagR* mutant occurred during this time interval, indicating that they both progressively colonized the plant and that the lack of induction of *pip* was not due to a growth deficiency of the *xagR* mutant in soybean (Fig. 4). These results suggest that the plant factor that enhances XagR function and, thus, *pip* expression is low in healthy plants but increases during the infection process.

Transcription of *xagR* is constitutive but XagR accumulates only in soybean plants.

Because *pip* transcription is apparently induced by specific molecules in living soybean plants and is dependent on *xagR*, we determined whether the transcription of *xagR* is itself also enhanced by the signal molecules. The *xagR* promoter was cloned upstream of the promoterless *inaZ* reporter gene to yield pXagR-KI which was introduced into both the *X. axonopodis* pv. *glycines* wild type and the *xagR* mutant strains. The ice nucleation activity of both strains was similar in all culture media and in planta, indicating that *xagR* was constitutively expressed, even in soybean plants (Supplementary Fig. S2A). The levels of *xagR* transcript measured by RT-PCR also did not differ under any condition. The constitutive expression of *xagR* differs from that of its homologs in *X. campestris* pv. *campestris* and *X. oryzae* pv. *oryzae* in that signal molecules from cab-

Fig. 4. A, Ice nucleation activity indicative of gene encoding proline iminopeptidase (*pip*) promoter activity in a wild-type strain of *Xanthomonas axonopodis* pv. *glycines* (circles) and an *xagR* mutant (triangles) harboring a *pip:inaZ* fusion at various times after infiltration into soybean leaves. **B,** Population size of the wild-type strain of *X. axonopodis* pv. *glycines* (circles) and an *xagR* mutant (triangles) at various times after infiltration. Vertical bars represent the standard error of mean log-transformed ice nucleation activity or population sizes.

Fig. 5. Western blot analysis of XagR in different strains of *Xanthomonas axonopodis* pv. *glycines* grown in minimal medium (left three lanes) or in media containing fresh soybean leaf macerates (left panel) or in uninoculated soybean leaves or leaves inoculated with the wild-type *X. axonopodis* pv. *glycines* strain 72 h after inoculation (right panel).

bage increased transcription of *xccR* (Ferluga and Venturi 2009; Ferluga et al. 2007) whereas *oryR* negatively regulated its own expression and functioned as a transcriptional regulator in the absence of rice signal molecules (Zhang et al. 2007).

The abundance of XagR was determined by Western blot analysis using anti-OryR antibodies to which it also could bind. XagR could not be detected in cells of either the wild type or the *xagR* mutant when grown in minimal media with or without added macerated soybean (Fig. 5). *xagR* was also overexpressed by placing it under the control of the *trp* promoter in pBBRMCS-5 and introducing it into *X. axonopodis* pv. *glycines* to create strain XagROX. XagR was readily detected in XagROX when grown in minimal media (Fig. 5). Importantly, abundant XagR was detected in *X. axonopodis* pv. *glycines* wild-type cells recovered from soybean plants 72 h or more after infiltration (Fig. 5). XagR was not detected in cells harvested from plants at either 24 or 48 h after inoculation (data not shown). Although the abundance of cells in plants 48 h after inoculation were two- to fivefold less than those present after 72 h (Fig. 4B), the presence of XagR in such cells should have been easily detected had it been produced at the concentrations seen in cells later in the infection process, suggesting that XagR accumulation increased substantially 48 h or more after inoculation, much like the induction of *pip* (Fig. 4A). These results indicated that some component or components in soybean that increased during the infection process facilitated accumulation of XagR, possibly by preventing its proteolytic degradation, suggesting that the modulation of XagR levels is a post-transcriptional process.

The *xagR* regulon in *X. axonopodis* pv. *glycines* 12-2.

To better understand the number and type of genes in *X. axonopodis* pv. *glycines* that are dependent on XagR for expression, the transcriptome of a wild-type *X. axonopodis* pv. *glycines* (in which XagR does not accumulate in culture) was compared with that of the *xagR* overexpression strain XagROX, in which XagR does accumulate. The abundance of transcripts of both *xagR* and *pip* as measured by RT-PCR were significantly higher in XagROX than in the wild-type strain (data not shown), probably accounting for the higher accumulation of XagR in XagROX. RNA-Seq analysis was performed on three independent replicate RNA samples collected from each strain grown to stationary phase in M9 minimal medium. cDNA was generated from mRNA-enriched total RNA preparations from

Fig. 6. Relative abundance of transcripts of various genes in strain XagROX in which *xagR* is overexpressed compared with that in wild-type *Xanthomonas axonopodis* pv. *glycines* in M9 media as determined by real-time reverse-transcriptase polymerase chain reaction. Vertical bars represent the standard error of mean ratio.

each strain and sequenced using an Illumina HiSeq 2000, with a minimum of 16,589,895 reads assignable to genes other than those in the ribosomal RNA operon (an average of 41 % of the total reads) for each replicate. Because the *X. axonopodis* pv. *glycines* 12-2 genome was not completely closed, the sequence reads were aligned to ORF present in an *X. axonopodis* pv. *glycines* 12-2 draft genome that was created for this study (Supplementary Tables S1 and S2).

Transcript levels of 79 ORF differed significantly and by at least fourfold between the wild-type strain and XagROX. Of these, 61 genes were more highly expressed in XagROX while 18 genes were expressed at a lower level compared with the wild-type strain (Supplementary Table S3). Although we could identify a conserved *lux* box in the promoter region of *pip*, we could not detect such elements in the promoter regions of other XagR-regulated genes. The transcript abundance of a subset of the XagR-regulated genes was also assessed using RT-PCR; relative levels of transcript abundance very similar to that observed by mRNA sequencing were seen for a given gene (Fig. 6), providing evidence that RNA sequencing yielded reliable quantitative estimates of transcript abundance in this study. The gene showing the highest induction in XagROX (932-fold) was *xagR* itself, confirming that, in our experimental conditions, *xagR* was indeed overexpressed. Likewise, as expected from the results of ice nucleation reporter gene assays and RT-PCR analysis, the *pip* transcript was 183-fold higher in XagROX than in the wild-type strain. Other genes highly up-regulated in XagROX included those encoding a membrane protease subunit (homolog XAC3964 from *X. axonopodis* pv. *citri* 306) and a protein similar to RtcB (homolog to XAC3965), as well as several genes with unknown function. Among the genes strongly downregulated in XagROX was a gene encoding a homolog of a *Yersinia* autotransporter protein H (YapH) (67-fold lower expression). YapH is predicted to be an autotransported adhesin that contributes to leaf attachment and virulence of *X. oryzae* pv. *oryzae* to rice (Das et al. 2009). A gene encoding a GAF/GGDEF/EAL domain protein and several proteins involved in general stress response were also negatively regulated by XagR.

XagR regulates the expression of a biosurfactant that contributes to swarming.

In addition to identifying genes regulated by XagR, we explored XagR-dependent phenotypes in culture that might not be easily linked to particular genes. XccR and Pip have been shown to be virulence factors in *X. campestris* pv. *campestris* that control the production of extracellular proteases, amylases, and the ability to spread and grow in the vascular system (Zhang et al. 2007). To explore whether XagR might also regulate such virulence factors, these traits were compared in the wild-type and XagROX strains in vitro. Although the expression of these traits did not differ between the two strains, overproduction of a biosurfactant was apparent in XagROX (Fig. 7). This was also associated with a concomitant increased ability of cells to spread over semisolid agar surfaces (Supplementary Fig. S3).

Contribution of XagR-regulated genes to the virulence of *X. axonopodis* pv. *glycines*.

Although we had found that *pip*, which is regulated by XagR, is required for full virulence of *X. axonopodis* pv. *glycines*, we also determined the contributions of other XagR-regulated genes. Thus, we disrupted the genes encoding the putative membrane protease subunit, the *rtcB* homolog, and the *yapH* homolog (designated XagID3706, XagID3707, and XagID2132, respectively) in *X. axonopodis* pv. *glycines*. The *rtcB* homolog mutant was significantly less virulent than the wild-type strain whereas the incidence of disease incited by

the XagID3706 mutant was similar to that of the wild-type strain. Interestingly, the incidence of disease caused by the *yapH* mutant was significantly greater than that incited by the wild-type strain on plants incubated in the greenhouse after inoculation (Table 1).

The role of *xagR* on bacterial attachment to plants and egression from lesions.

Given that a mutant of *yapH*, which was downregulated in cells of XagROX in which XagR had accumulated, was hyper-virulent to soybean, we addressed whether this trait was due to contributions of this protein to the adhesiveness of *X. axonopodis* pv. *glycines*. In principle, cells with low adhesiveness might more readily invade leaves and initiate infection events than cells that are more adhesive. To quantify the role of XagR and YapH in attachment to abiotic surfaces, the adhesion of cells to polystyrene microtiter plates was assessed. The number of adherent cells of the *yapH* mutant and XagROX as measured by crystal violet staining after 7 h of incubation was lower than the wild-type strain; the differences in adhesion were largest when observed 24 h after inoculation (Fig. 8A). The adhesion of these strains to the surface of soybean leaves also was studied by quantifying those cells remaining on leaves after they were dipped into bacterial cell suspensions. The number of cells of the *yapH* mutant and of XagROX that remained attached after gentle washing of the inoculated leaves was 10- to 100-fold lower than and differed significantly from that of the wild-type strain (Fig. 8B). Thus, XagR accumulation apparently reduces the amount of YapH available to participate in the adhesion of the cells to the surface of soybean leaves.

Because YapH contributes to attachment of *X. axonopodis* pv. *glycines* to the surface of soybean leaves, we reasoned that it might also be involved in cell retention within the apoplast of infected leaves. We assessed the ability of *X. axonopodis* pv. *glycines* to egress from the apoplast by measuring the number of cells released from the edges of leaf discs cut from surface-sterilized, infected leaves. Although the numbers of cells of the *yapH* mutant and XagROX released from the tissue were consistently higher than that of the wild-type strain, these differences were not statistically significant. Large differences would not have been expected because XagR presumably had accumulated to high levels in the wild-type strain in the in-

fected plants and, thus, YapH levels would be expected to be low in all of these strains. However, significantly fewer cells of the *xagR* mutant escaped the discs compared with the wild-type strain (Fig. 8C). These results suggest that XagR plays a role in the disease process by suppressing YapH expression and, thus, promoting *X. axonopodis* pv. *glycines* egression from the apoplast onto the leaf surface, where it could be spread to initiate new infections.

Although YapH-mediated cell adhesion affected the apparent virulence of *X. axonopodis* pv. *glycines*, such an effect would be expected to be context dependent. Under conditions when removal of cells from a leaf by rainfall would not be expected (such as in our initial greenhouse experiments), cells of lower adhesiveness might more easily invade leaves, while loss of adhesiveness would be expected to lead to removal of cells from leaf surfaces during rainfall events, thus lowering infection events. Thus, we compared the role of XagR-mediated changes in adhesiveness on virulence of *X. axonopodis* pv. *glycines* under conditions of artificial rain following topical inoculation compared with relatively dry conditions in greenhouse experiments. Strains XagROX and the *yapH* mutant, which were less adherent to soybean leaves, both incited significantly fewer lesions than the wild-type strain when leaves were exposed to simulated rainfall 30 min after inoculation (Fig. 9). As observed previously, the *yapH* mutant was somewhat more virulent while XagROX was less virulent than the wild-type strain when rainfall was not applied after inoculation (Fig. 9).

DISCUSSION

In this study, we have analyzed the dynamics of *X. axonopodis* pv. *glycines* interactions with soybean during the infection process, with particular attention to the role of the transcriptional regulator XagR. The primary structure of XagR is similar to that of OryR and XccR, having a typical modular structure of other LuxR-family response regulators involved in QS with an inducer-binding domain in the N terminus contains and a helix-turn-helix DNA-binding domain in the C terminus. Although *xagR* is constitutively expressed both in culture and in planta, XagR only accumulates in cells in infected plants. Although XagR was not stabilized by any of the structurally different AHL tested, unidentified compounds in the plant may

Fig. 7. Visible halos of surfactant produced by various strains of *Xanthomonas axonopodis* pv. *glycines* as visualized by an atomized oil assay after growth for 3 days.

enable its accumulation, apparently in a way similar to that seen for OryR (Ferluga and Venturi 2009). The plant factors that activate XagR are apparently not host plant specific because some induction of *pip* was observed when *X. axonopodis* pv. *glycines* was infiltrated into rice and cabbage as well as soybean. In contrast to studies of OryR, however, no XagR function, as measured by the activation of *pip*, could be observed in vitro with extracts of any plants, including rice, suggesting that the inducing compounds for OryR and XagR differ. PsoR, a LuxR homolog in *P. fluorescens* CHA0, was recently shown to be stabilized by rice and wheat extracts but not by cucumber extracts (Subramoni and Venturi 2009), suggesting that there is host specificity in such factors. To date, the identity of these plant compounds remains unknown. Those from rice and cabbage that interact with OryR and

XccR are suggested to be small molecules (<1 kDa) (Ferluga and Venturi 2009; Wang et al. 2011). Although the signal molecules in rice and cabbage are apparently relatively abundant even in uninfected plants, the compounds in soybean seem to accumulate only after infection, because little induction of *pip* is seen for at least 24 to 48 h after inoculation and does not reach a maximum until 72 h or more (Fig. 4). Identification of the plant compound needed for stabilization of XagR should help unravel the complex interactions that occur during infection events.

Both XagR and Pip are required for full virulence of *X. axonopodis* pv. *glycines* to soybean, and XagR is required for *pip* expression similar to that seen for OryR and XccR in *X. oryzae* pv. *oryzae* and *X. campestris* pv. *campestris*. The role of Pip in virulence remains unclear. Pip catalyzes the removal of N-terminal prolines from peptides with fairly high specificity. D-alanine is also removed, with much lower efficiency than the L-isomer (Alonso and Garcia 1996). In *X. campestris* pv. *campestris*, Pip is a periplasmic protein and might hydrolyze some small molecules from the host plant (Zhang et al. 2009). In *X. oryzae* pv. *oryzae*, the activity of the *pip* promoter in media containing extracts of infected rice (10 days after inoculation) was somewhat higher than that containing uninfected rice macerates (Ferluga and Venturi 2009), suggesting that its induction might be associated with adaptation to the presence of inhibitory compounds made by the plant during infection. The delayed induction of *pip* in *X. axonopodis* pv. *glycines* in soybean until many hours after inoculation strongly suggests that its role might be to respond to increases in defensive compounds that occur during the infection event. Although XagR controls at least 79 genes, given that *xagR* and *pip* mutants exhibit similarly reduced virulence to soybean, it appears that Pip contributes substantially to the virulence phenotypes regulated by XagR.

Compounds found in infected soybean likely bind to and, thus, stabilize XagR, although its pattern of transcriptional regulation appears to differ from that of related proteins. Many LuxR regulators are insoluble or unstable and susceptible to proteolytic degradation unless they bind to their cognate AHL. TraR of *A. tumefaciens*, a member of the LuxR family, required 3-oxo-octanoyl homoserine lactone-mediated folding for resistance to cytoplasmic proteases (Zhu and Winans 1999) and dimerization also enhances its resistance to cellular proteases (Pinto and Winans 2009). The expression of *xccR* in *X. campestris* pv. *campestris* was repressed in culture media by XerR, an NtrC-type response regulator which was relieved

Fig. 8. A, Attachment of various strains of *Xanthomonas axonopodis* pv. *glycines* to polystyrene wells assessed by crystal violet staining of adherent cells after 5 min, 7 h, and 24 h of incubation; **B**, attachment of the strains to the surface of soybean leaves after topical application. **C**, Egression of the strains from the edges of discs of infected soybean leaves.

Fig. 9. Incidence of bacterial pustule disease on soybean incited by different strains of *Xanthomonas axonopodis* pv. *glycines* after spray inoculation when plants were allowed to dry slowly after inoculation (solid bars) or subjected to 30 min of simulated rain after inoculation (open bars). Vertical bars represent the standard error of mean disease incidence per plant.

when the bacteria were grown in planta (Wang et al. 2011). In *X. oryzae* pv. *oryzae*, OryR negatively regulated its own transcription but its expression was independent of the presence of rice extract (Ferluga and Venturi 2009). However, accumulation of OryR occurred in the presence of a rice signal molecule (Ferluga et al. 2007). In this study, we found that *xagR* transcription was constitutive but that XagR accumulated only in soybean plants. Interestingly, although *xagR* was expressed both in culture and in planta, XagR was detected only in cells recovered from soybean plants 72 h or more after infiltration or in minimal media when *xagR* was overexpressed in trans. Although the number of *X. axonopodis* pv. *glycines* cells present in plants soon after inoculation is relatively low, we found no evidence of XagR in cells at early stages of infection, suggesting that it accumulates relatively late in the infection process. This suggests that a signal molecule that accumulates in soybean only during the infection process has two effects: i) by binding to XagR, it indirectly controls XagR abundance, probably by altering its proteolytic degradation; and ii) by binding XagR, it activates it to bind DNA, probably by forming a dimer, thereby affecting expression of target genes. Interestingly, the virulence of XagROX, in which XagR does accumulate, was very low in soybean, suggesting that early accumulation of XagR during the infection process is inhibitory. Given the relatively large number of genes regulated by XagR, it remains unclear which ones might inhibit the infection process if prematurely expressed.

XagR apparently regulates a large number of genes directly or indirectly because 44% of the annotated genes of *X. axonopodis* pv. *glycines* were differentially expressed in its presence ($P = 0.01$). Estimates of transcript abundance obtained by RNA sequencing with the Illumina HiSeq 2000 System correlated well with those made by quantitative RT-PCR. Noteworthy genes positively regulated by XagR include the *pip* gene that is located immediately downstream of *xagR* and which contains a putative *lux* box in the intergenic region with *xagR* (Fig. 1). Similar to that shown for XccR and OryR, XagR likely binds to a low molecular weight plant compound and, subsequently, regulates *pip* expression via its well-conserved *lux* box. In contrast, the *pip* gene of the biocontrol strain *P. fluorescens* CHA0, which is adjacent to the *xagR* homolog *psrR*, does not have a *lux* box and is not regulated by *psrR* (Subramoni and Venturi 2009); the *pip* gene in *P. fluorescens* likewise also lacks a *lux* box (Subramoni and Venturi 2009). Although the mechanism by which Pip contributes to the virulence of *Xanthomonas* pathogens remains unknown, LuxR-type regulators appear to play a direct role in their regulation. The fact that well-conserved *lux* boxes were not apparent in the promoter regions of the other 78 genes regulated by XagR suggests that they are indirectly regulated but does not exclude the possibility of direct regulation because *lux* boxes can exhibit substantial variability. The predicted gene coding for a membrane protease related to stomatin or prohibitin (XAC3964 homolog from *X. axonopodis* pv. *citri* 306) and *rtcB* (homolog to XAC3965) also exhibited substantial increases in transcript levels in XagROX. The membrane protease may contribute to post-translational modification of other proteins, perhaps in a manner similar to that proposed for Pip (Kihara et al. 1996). RtcB enzymes are novel RNA ligases that join 2',3'-cyclic phosphate and 5'-OH ends of RNA that might afford bacteria a means to recover from stress-induced damage (Tanaka et al. 2011). Although no function of these genes has been reported in bacterial plant pathogens, the disruption of either of them significantly reduced the virulence of *X. axonopodis* pv. *glycines*. Four genes in an operon defined by XagID 2143 to 2146 encoding exoenzyme regulatory protein AepA, membrane protein 2 having some similarity to the thiosulphate:quinone oxi-

doreductase DoxD, an isochorismatase family protein, and a putative transcriptional regulator in the AraC family, respectively, were also upregulated by XagR. The proteins belonging to the AraC family of transcriptional regulators have three main regulatory functions in common: carbon metabolism, stress response, and pathogenesis (Gallegos et al. 1993). Again, because they presumably are expressed primarily late in the infection process, they may be required mainly to deal with events that occur after initial plant interactions.

We observed upregulation of several genes that mediate responses to low water availability, including the choline dehydrogenase and betaine aldehyde dehydrogenase loci as well as the potassium-transporting ATPase operon, in strain XagROX compared with the wild-type strain. Increases in the osmolality of bacterial environments usually result in changes in intracellular solute concentration and cell volume (Poolman and Glaeser 1998). To cope with such stresses, bacteria accumulate osmotically active solutes, including proline, glutamic acid, glutamine, α -aminobutyric acid, ectoine, betaine, and potassium (Galinski and Truper 1994). Choline dehydrogenase catalyzes the oxidation of choline to glycine betaine, with betaine aldehyde as an intermediate (Landfald and Strom 1986). Numerous in vitro studies have shown that betaine acts as an osmoprotectant by stabilizing both the quaternary structure of proteins and membrane integrity against the adverse effects of high salinity and extreme temperatures (Le Rudulier et al. 1984). Plant restriction of water availability has been demonstrated in both compatible and incompatible interactions of plant-pathogenic bacteria with plants (Beattie 2011). Therefore, it is possible that *X. axonopodis* pv. *glycines* anticipates the lower water availability that might accompany later stages of infection of soybean, with the accumulation of XagR triggering the accumulation of osmoprotectants.

Interestingly, the expression of *yapH* and a GAF/GGDEF/EAL domain protein were both downregulated in XagROX compared with the wild-type strain. GGDEF-domain proteins are involved in the synthesis of cyclic di-GMP, which is a second messenger in bacteria that regulates a range of external cellular functions such as motility and adhesion that often contribute to the virulence of pathogens (Hengge 2009; Simm et al. 2004; Tamayo et al. 2007). Therefore, the modulation of *yapH* expression might have been via alteration of cyclic di-GMP signaling. YapH was required for adhesion of the vascular pathogen *X. fuscans* subsp. *fuscans* to seeds, leaves, and abiotic surfaces but not for in planta transmission to seeds or for infection of bean leaves (Darsonval et al. 2009); thus, *yapH* was considered a potential antivirulence gene (Foreman-Wykert and Miller 2003). *yapH* mutants of *X. oryzae* pv. *oryzae* also exhibit deficiencies in leaf attachment or entry into rice xylem vessels (Das et al. 2009). Importantly, we also found that a *yapH* mutant and XagROX were deficient in adhesion to both abiotic surfaces and soybean leaf surfaces. Because XagR does not accumulate in cells in culture, we were not surprised that cultured cells of the *xagR* mutant did not differ from the wild-type strain in adhesiveness. In contrast, although egress of the *yapH* mutant and XagROX from infected leaves was only slightly greater than that of the wild-type strain, the *xagR* mutant was much more strongly retained in the infected tissue compared with the wild-type strain. In infected leaves, XagR would have accumulated and suppressed the expression of YapH, whereas no such repression of YapH would have occurred in the *xagR* mutant, leading to a greater adhesiveness.

The virulence contribution of *yapH* and other genes in *X. axonopodis* pv. *glycines* under the control of XagR is strongly context dependent. Indeed, we observed that disruption of *yapH* increased the ability of *X. axonopodis* pv. *glycines* to cause leaf lesions on soybean under relatively dry conditions but, under

wet conditions in which simulated rain was applied to plants after inoculation, the *yapH* mutant incited significantly fewer lesions compared with the wild-type strain (Fig. 9). It is easy to imagine that the lower adhesiveness of the *yapH* mutant allows it to more easily move on moist leaves (not experiencing rain) after inoculation; because the bacteria are not immobilized by attachment to the leaf surface, they would have more opportunities to enter stomata and begin intercellular growth, leading eventually to lesions. In contrast, such a strain would be more easily washed from leaves during rainfall events, thereby removing the *yapH* mutant from the infection court.

Considerations of the temporal pattern of accumulation of XagR during the infection process, and the traits that are under the control of XagR, enable us to develop a plausible model for the role of this LuxR regulator in the cycle of bacterial pustule disease of soybean (Fig. 10). Upon arrival at a healthy plant, *X. axonopodis* pv. *glycines* apparently does not perceive any plant signals that would enable the accumulation of XagR. Under such conditions, *pip* is not induced and adhesins such as YapH are not suppressed, leading to an adhesive state, allowing the strong attachment of cells to the leaf surface, thereby becoming resistant to removal by rain. Those cells that enter the leaf begin to grow in the apoplast and, after 2 or more days, the plant has accumulated enough of a signal molecule that is produced during the infection process that XagR is stabilized and accumulates in cells, triggering numerous changes in gene expression such as *pip* induction and *yapH* repression. Genes associated with biosurfactant production are also induced at this time. Such intercellular cells would be of low adhesiveness and the surfactants would enable the cells to spread within the apoplast, thereby expanding the lesion and increasing population size. Such cells would also be more capable of egression from the plant via the pustules that form on leaves.

Upon escape from the plant and perhaps rain splash to other leaves, the cells would initially be of low adhesiveness, enabling them to invade new leaves. However, the plant factor to which the cells had responded within infection sites would be diluted and XagR might quickly be degraded, enabling the restoration of an adhesive state and the suppression of virulence genes such as *pip* and others that are useful or required only in later stages of plant infection. Thus, perhaps distinct from other LuxR regulators, XagR seems to play a particularly large role in coordinating the temporally distinct phases of the life cycle of this pathogen. That is, phenotypes that are beneficial in one setting can be temporally regulated to avoid conflicts via such a regulator which appears to sense different stages in the infection process.

MATERIALS AND METHODS

Bacterial strains, plasmids, and recombinant techniques.

Bacterial strains and plasmids used in this study are described in Table 2. *X. axonopodis* pv. *glycines* strains were cultured at 28°C in LB agar (LA) or in the minimal medium M9 (Sambrook et al. 1989). Cells were also cultured on media containing soybean extracts, including i) fresh soybean medium consisting of 10 g of soybean leaves powdered in liquid nitrogen and added to 100 ml of sterilized distilled water and ii) autoclaved soybean medium consisting of fresh soybean medium that was autoclaved. Bacterial growth was monitored by measuring the absorbance of cell suspensions at 600 nm. Antibiotics were added to media at the following concentrations: kanamycin, 50 µg/ml; chephalexin, 50 µg/ml; and gentamycin, 40 µg/ml. All DNA manipulations, including DNA isolation, plasmid extraction, restriction digestion, ligation, and gel electrophoresis, were performed as described previously (Sambrook et al. 1989).

Fig. 10. Model of the temporal regulation of different traits of *Xanthomonas axonopodis* pv. *glycines* that are regulated by XagR in relation to the process of disease development in a population of plants. Circles represent the putative signal molecules from the host.

Genomic sequencing and annotation.

Genomic DNA was prepared from a culture of *X. axonopodis* pv. *glycines* 12-2 grown in nitrogen yeast glycerol broth medium using a Qiagen genomic DNA purification kit (Qiagen Inc., Valencia, CA, U.S.A.). Whole-genome sequencing was performed on a 454 GS-FLX Titanium sequencer in accordance with the manufacturer's protocol at Kansas State University, and Illumina Solexa GA sequencing at the Functional Genomics Laboratory at the University of California, Berkeley. Paired-end reads (approximately 400 and 100 bp, respectively) were generated in both cases. All reads were mapped to the genome of *X. axonopodis* pv. *citri* 360 as a reference to obtain an *X. axonopodis* pv. *glycines* consensus sequence. All short reads that were not mapped to the reference genome were assembled de novo using CLCbio Genomics Workbench version 4.0, with length fraction and similarity set at 0.8 and all other parameters using default values. The *X. axonopodis* pv. *glycines* consensus sequence and contigs were submitted to the Rapid Annotations using Subsystems Technology server (Aziz et al. 2008) to identify protein-encoding, rRNA, and tRNA genes and to assign functions to these genes. The draft genome is described in Supplementary Table S5.

Generation of XagR overexpressing strain.

The XagR-overexpressing strain XagROX was constructed by placing the coding region of *xagR* downstream of the strong *Escherichia coli* *trp* operon promoter in pBBR1MCS-5 and introducing it into *X. axonopodis* pv. *glycines* 12-2. *xagR* was amplified using *X. axonopodis* pv. *glycines* genomic DNA as the template with primers Ptp-F-1, Ptp-F-2, Ptp-F-blunt, and

XagR-R-blunt (Supplementary Table S4) and cloned into the *Sma*I site of pBBR1MCS-5 to yield pTrp::xagR, which was then introduced into *X. axonopodis* pv. *glycines* by electroporation.

RNA isolation and quantitative RT-PCR analysis.

Total RNA was extracted using the Qiagen RNA/DNA Mini Kit, and DNA was removed using an on-column DNase treatment (RNeasy Mini Kit with DNase I; Qiagen Inc.). RNA samples were analyzed for quality using a BioAnalyzer 2100 (Agilent, Palo Alto, CA, U.S.A.). cDNA was generated from 1 µg of RNA using SuperScript II (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) and random hexamers. To confirm that DNA was removed, samples processed in parallel without reverse transcriptase served as negative controls in quantitative (Q)-PCR experiments described below. Q-PCR was performed on 1 µg of the cDNA using LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche, Indianapolis, IN, U.S.A.) on a Roche Lightcycler II (Roche) following the manufacturer's specifications. An external standard curve was generated using purified *ihfA* (integration host factor A) DNA (Champoiseau et al. 2006). Melting curve analysis was used to verify amplification of a single product. The concentration of amplification products from negative controls (RNA samples to which no superscript was added) was undetectable in all cases, indicating a lack of interference from contaminating DNA.

mRNA sequencing.

Sequencing was performed on mRNA isolated from *X. axonopodis* pv. *glycines* wild-type strain 12-2 and XagR-overexpressing strain XagROX. Total RNA was isolated with TRIzol

Table 2. Bacterial strains and plasmids

Bacterial strain or plasmid	Relevant characteristic ^a	Reference or source
<i>Xanthomonas axonopodis</i> pv. <i>glycines</i> 12-2	Wild type, soybean pathogen	Thowthampitak et al. 2008
<i>xagR</i> mutant	Km ^r , <i>xagR</i> ::Kan, 12-2 derivative	This study
<i>pip</i> mutant	Km ^r , <i>pip</i> ::Kan, 12-2 derivative	This study
<i>xagR/pip</i> double mutant	Km ^r , <i>xagR</i> and <i>pip</i> ::Kan, 12-2 derivative	This study
XagID3706	Km ^r , homolog to XAC3964 from <i>Xac</i> ::Kan, 12-2 derivative	This study
XagID3707	Km ^r , homolog to XAC3965 from <i>Xac</i> ::Kan, 12-2 derivative	This study
XagID2132	Km ^r , <i>yapH</i> ::Kan, 12-2 derivative	This study
XagROX	Wild type containing <i>xagR</i> fusion with <i>trp</i> promoter cloned into pBBR1MCS-5	This study
XagR ⁺	<i>xagR</i> mutant complemented with pBBR::xagR	This study
XagKI- <i>xagR</i>	Wild type, containing <i>xagR</i> promoter cloned with <i>Sac</i> I in pPROBE-KI	This study
XagKI- <i>pip</i>	Wild type, containing <i>pip</i> promoter cloned with <i>Sac</i> I in pPROBE-KI	This study
XagRKI- <i>xagR</i>	<i>xagR</i> mutant, containing <i>xagR</i> promoter cloned with <i>Sac</i> I in pPROBE-KI	This study
XagRKI- <i>pip</i>	<i>xagR</i> mutant, containing <i>pip</i> promoter cloned with <i>Sac</i> I in pPROBE-KI	This study
Plasmids		
pTok2	ColE1 replicon, suicide plasmid, Tc ^r	Kitten and Willis 1996
pPROBE-KI	Broad-host-range plasmid with a transcriptional fusion cassette containing a promoterless <i>inaZ</i> reporter gene; Km ^r	Miller et al. 2000
pJR4	<i>sacB</i> ⁺ and <i>flp</i> ⁺ (Cro promoter) from pFLP2 cloned into pUFR47. IncW, Mob ⁺ , <i>lacZ</i> α ⁺ , Par ⁺ , <i>sacB</i> ⁺ , <i>flp</i> ⁺ (Cro promoter), Gm ^r , Ap ^r	Castañeda et al. 2005
pKD13	FRT-Kan-FRT, oriR6K, Ap ^r , Km ^r	Datsenko and Wanner 2000
pBBR1MCS-5	Broad host range cloning vector, <i>lacZ</i> , Gm ^r	Kovach et al. 1995
pTok2::Δ <i>xagR</i>	Δ <i>xagR</i> ::Kan from overlapping PCR cloned into pTok2, Tc ^r , Km ^r	This study
pTok2::Δ <i>pip</i>	Δ <i>pip</i> ::Kan from overlapping PCR cloned into pTok2, Tc ^r , Km ^r	This study
pTok2::Δ <i>xagR-pip</i>	Δ <i>xagR-pip</i> ::Kan from overlapping PCR cloned into pTok2, Tc ^r , Km ^r	This study
pTok2::Δ homolog to XAC3964	ΔHomolog to XAC3964::Kan from overlapping PCR cloned into pTok2, Tc ^r , Km ^r	This study
pTok2::Δ homolog to XAC3965	ΔHomolog to XAC3965::Kan from overlapping PCR cloned into pTok2, Tc ^r , Km ^r	This study
pTok2::Δ <i>yapH</i>	Δ <i>yapH</i> ::Kan from overlapping PCR cloned into pTok2, Tc ^r , Km ^r	This study
pTok2::Δ homolog to XAC0824	ΔHomolog to XAC0824::Kan from overlapping PCR cloned into pTok2, Tc ^r , Km ^r	This study
pXagR-KI	<i>xagR</i> promoter cloned with <i>Sac</i> I in pPROBE-KI	This study
pPip-KI	<i>pip</i> promoter cloned with <i>Sac</i> I in pPROBE-KI	This study
pTrp::xagR	<i>xagR</i> gene fusion with <i>trp</i> promoter cloned into pBBR1MCS-5, <i>lacZ</i> , Gm ^r	This study
pBBR::xagR	<i>xagR</i> gene with native promoter cloned into pBBR1MCS-5, <i>lacZ</i> , Gm ^r	This study

^a Km^r, Tc^r, Gm^r, and Ap^r indicate kanamycin, tetracycline, gentamycin, and ampicillin resistance, respectively.

(Invitrogen Life Technologies) from cells grown in M9 minimal medium using the method of Santiago-Vazquez and associates (2006). Contaminating DNA was removed using the Turbo DNA-free kit (Ambion, Austin, TX, U.S.A.) and was cleaned using the RNeasy mini kit (Qiagen Inc., Basel, Switzerland). 16S and 23S rRNA were removed from total RNA using the Ribo-Zero rRNA Removal Kit (gram-negative bacteria) (Epicentre, Madison, WI, U.S.A.) according to the manufacturer's protocol, with 5 µg of total RNA treated per depletion reaction. Enriched mRNA samples were assessed with a 2100 Bioanalyzer (Agilent, Santa Clara, CA, U.S.A.) to confirm the removal of 16S and 23S rRNA. Quantification of remaining mRNA prior to preparation of cDNA fragment libraries was performed using the qBit RNA assay (Invitrogen Life Technologies). Ambion RNA fragmentation reagents (number AM8740; Ambion) were used to generate 60- to 200-nucleotide RNA fragments with an input of 100 ng of mRNA. Fragmented RNA was purified using ethanol precipitation in the presence of glycogen and precipitated RNA was resuspended in RNase-free water. First-strand cDNA synthesis was performed using random primers (Invitrogen Life Technologies) and Superscript II Reverse Transcriptase, followed by second-strand cDNA synthesis using RNaseH and DNA pol I (Invitrogen Life Technologies). Double-stranded cDNA was purified using AMPure XP beads (Beckman Coulter) with PEG 6000 added to a final concentration of 6.5% (wt/vol). cDNA end repair was performed using the Klenow DNA polymerase, T4 DNA polymerase, and T4 polynucleotide kinase (all New England Biolabs, Beverly, MA, U.S.A.). An A tail was added to DNA fragments using Klenow exo minus and 250 µM (final concentration) dATP. Illumina adapters were ligated to the DNA fragments using T4 DNA ligase, with PEG 6000 addition (5.0% final concentration) (New England Biolabs). Fragments were amplified using Illumina barcoded primers and Phusion DNA polymerase (New England Biolabs). Amplified fragments were purified similarly as above, except without PEG addition. Fragment sizes of 200 to 300 nucleotides were confirmed using the 2100 Bioanalyzer. Sequencing was performed by running 36 cycles on the Illumina HiSeq 2000. Amplified materials from each of three replicate samples were assigned distinct bar-coded linkers and loaded onto independent flow cells. All short reads of cDNA sequence were mapped to *X. axonopodis* pv. *glycines* ORF using Bowtie 0.12.7 (Langmead et al. 2009). Differential gene expression was assessed with edgeR, a Bioconductor package (Gentleman et al. 2004; Robinson et al. 2010).

Knockout of genes regulated by XagR.

Individual targeted disruptions of *xagR*, *pip*, *yapH*, and the genes homologous to *X. axonopodis* pv. *citri* loci 3964 and 3965 were accomplished using overlap extension mutagenesis. The upstream and downstream regions flanking target genes in *X. axonopodis* pv. *glycines* were amplified using primers unique to these regions, with one having an extension complementary to the kanamycin resistance cassette from pKD13 to generate two amplicons with ends overlapping those of the resistance cassette (Datsenko and Wanner 2000). Overlap extension PCR was used to link the two PCR amplicons and the resistance cassette; this larger fragment was then cloned into the destination vector pTok2 using the quick ligation protocol (New England Biolabs Inc.) and introduced into the mobilizing strain *E. coli* S17-1 by transformation, then transferred to *X. axonopodis* pv. *glycines* via conjugation, selecting for transconjugants on LA containing kanamycin and cephalixin. Because pTok2 is nonreplicative in *Xanthomonas* spp., kanamycin-resistant but tetracycline-sensitive colonies of *X. axonopodis* pv. *glycines* were expected to have undergone a double-cross-

over event between the DNA cloned in pTok2 and the corresponding sequence in the *X. axonopodis* pv. *glycines* chromosome, resulting in deletion of the target gene. Gene disruption was confirmed using PCR, with primers specific to the sequences flanking each gene.

Complementation of *xagR* mutants.

To complement the *xagR* mutant, the 1.4-kb sequence of *xagR* containing the native promoter was amplified using primers XagRcom-F (*Sall*) and XagRcom-R (*HindIII*) and the amplicon was then digested with both *Sall* and *HindIII* and ligated into the multiple cloning site of vector pBBR1MCS-5 to yield pBBR::xagR, which was then introduced into *xagR* mutants by electroporation.

inaZ-reporter gene fusion assays.

The promoter-containing sequences upstream of *xagR* and *pip* were amplified from genomic DNA of *X. axonopodis* pv. *glycines* using primers XagRPRO-F (*SacI*) and XagRPRO-R (*SacI*), and PipPRO-F (*SacI*) and PipPRO-R (*SacI*), respectively. Each amplicon was digested with *SacI* (New England Biolabs), purified using a QIAEX II gel extraction kit (Qiagen Inc.), and cloned immediately 5' to the promoterless *inaZ* ice nucleation reporter gene in the broad-host-range vector pPROBE-KI (Miller et al. 2000). The resulting plasmids, designated pXagR-KI and pPip-KI, were transformed into competent cells of *E. coli* DH5α (Sambrook et al. 1989) and introduced into *X. axonopodis* pv. *glycines* as well as the *xagR* mutant of *X. axonopodis* pv. *glycines* by electroporation with a Bio-Rad GenePulser Xcell electroporation system (Hercules, CA, U.S.A.).

The ice nucleation activity of cells harboring pXagR-KI and pPip-KI in vitro was determined after growth for 2 days at 25°C in M9 medium with or without addition of fresh or autoclaved soybean extract (10%) after suspension in 10 mM potassium phosphate buffer, pH 7.4. In planta ice nucleation activity was assessed in cells recovered at various times from inoculated plants. Cells were harvested from LA, resuspended to a final density of approximately 10⁸ cells/ml, and infiltrated under vacuum into the intercellular spaces of soybean plants (3 to 4 weeks old). Plants were incubated under light (12-h photoperiod) at 25°C and replicate samples of individual leaves were macerated in buffer. The ice nucleation activity of serial dilutions of either bacterial cell suspensions or leaf macerates was measured by a droplet freezing assay performed at -5°C as described previously (Loper and Lindow 1996). Ice nucleation activity was normalized for the number of viable cells present in a sample as determined by dilution plating on LA amended with kanamycin. The experiments were repeated three to five times with similar results.

Western blot analysis.

Bacterial cells from 10 ml of overnight shake cultures were pelleted by centrifugation for 10 min at 14,000 × g and then resuspended in 0.75 ml of lysis buffer (50 mM Tris [pH 8.0], 10% glycerol, 0.1% Triton X-100, and Complete Mini protease inhibitors cocktail) (Roche). Cells were disrupted by sonication (Vibracell; Sonics Materials Inc., Newton, CT, U.S.A.) and 6 µg of total protein (as determined by a Bradford protein assay) was mixed 4:1 with sample buffer (0.1 M Tris [pH 8.3], 50% glycerol, 10% sodium dodecyl sulfate [SDS], 25% β-mercaptoethanol, and traces of bromophenol blue) and separated by SDS polyacrylamide gel electrophoresis (PAGE) (15% [wt/vol] polyacrylamide). Bacterial cells were also recovered from infected soybean plants. Individual leaves of plants vacuum infiltrated were harvested at different times after inoculation and macerated in phosphate buffer. Total pro-

tein content of the plant macerate was performed by boiling the protein suspension in sample buffer for 10 min. Equal amounts of total protein (approximately 6 µg) were then separated by SDS-PAGE as previously outlined. In both cases, proteins were transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA, U.S.A.) using a tank system according to the manufacturer's instructions. The membrane was subjected to Western blot analysis using polyclonal antibodies (1:5000 dilution) against OryR, the LuxR homolog of *X. oryzae* pv. *oryzae*, provided by Vittorio Venturi (Ferluga and Venturi 2009; Ferluga et al. 2007) and an HRP conjugated goat anti-rabbit secondary antibody (Promega Corp., Madison, WI, U.S.A.). The membrane was developed using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, U.S.A.) and blue x-ray film (Phenix Research Products, Candler, NC, U.S.A.).

Biosurfactant detection.

Biosurfactant production of *X. axonopodis* pv. *glycines* was assessed on semisolid LA plates containing 0.4% technical agar, as described previously (Burch et al. 2010). Cells were grown overnight on LA medium, then harvested and washed in potassium phosphate buffer. Cells were resuspended in buffer to a concentration of 10^8 cells/ml and then were spotted (10 µl) onto a plate and incubated for 3 days at room temperature. The biosurfactant was detected by an atomized oil assay in which biosurfactant halos are visualized with an indirect source of bright light (Burch et al. 2010). The radii of biosurfactant spreading was measured (from the leading edge of the bacterial colony to the edge of the surfactant halo). The swarming distance was calculated as the average diameter of spreading colonies initiated from point sources, as above.

Bacterial adhesion assays.

To assess bacterial adhesion to abiotic surfaces, cells of overnight cultures in LB were resuspended to a concentration of 10^7 cells/ml in M9 minimal medium, and 100 µl was incubated individually in wells of 96-well flat-bottom polystyrene tissue culture plates and incubated at 28°C for different lengths of times before the medium was removed using a pipette. The wells were washed gently three times with 150 µl of sterile distilled water, and the plates were transferred to a 60°C incubator for 20 min. The abundance of the surface-attached biofilm was determined by crystal violet staining as in other studies (Davey and O'Toole 2000). Dye abundance was measured by absorption at 570 nm using a microtiter plate reader. Readings from 36 replicate wells were averaged. The experiments were repeated three times with similar results.

Bacterial adhesion to soybean leaves was assessed by immersing six individual leaves into 500 ml of a suspension of a given bacterial strain (10^7 cells/ml) at 28°C. After 5 min, 3 h, and 7 h, the leaves were removed and rinsed gently with distilled water for 30 s. To enumerate the attached bacteria, a single 2-cm-diameter disc was cut from a portion of each leaf in an area lacking major veins using a cork borer, the discs were homogenized using a mortar and pestle, and cells were enumerated by dilution plating.

Bacterial egression assay.

Bacterial suspensions (10^7 cells/ml) in 10 mM phosphate buffer were vacuum infiltrated into soybean plants and incubated at 28°C for 4 days. Six leaves from plants in each treatment were then detached and surface sterilized using UV irradiation (254 nm, with flux of approximately 1,000 ergs/m²/s for 30 min each side). A 2-cm disc was cut from each leaf as above and was placed into a tube containing 1 ml of sterile distilled water and incubated at 28°C for 30 min without

agitation, and the egressing bacteria were enumerated by dilution plating.

Virulence and bacterial growth in soybean.

The virulence of *X. axonopodis* pv. *glycines* was assessed on soybean 'Spencer' following topical spray application (Kaewnum et al. 2005). Briefly, cell suspensions of a given strain (optical density at 600 nm = 0.2; approximately 10^8 cells/ml) in 1 mM buffer were sprayed onto leaves of plants (approximately 6 weeks old) maintained in a greenhouse (average temperature approximately 25°C). For the first 24 h after inoculation, plants were held in an enclosed plastic tent to maintain high humidity and moisture on leaves before being returned to the greenhouse bench. In an experiment designed to simulate rainfall, plants were sprinkled with distilled water (approximately 500 ml/plant) over a period of approximately 30 min immediately after spray inoculation. At 7 to 10 days after inoculation, disease severity was assessed by counting the number of lesions on each leaf. Three trifoliate leaves (one each collected from the top, middle, and basal portion of three plants) from each of five replicate pots were evaluated for each strain. The experiments were repeated three times with similar results.

Bacterial growth was assessed within leaves in which bacteria had been infiltrated under vacuum. Briefly, cells of a given strain were resuspended in phosphate buffer and their concentrations determined from their optical density at 600 nm, then diluted to a final density of approximately 10^6 cells/ml and infiltrated under vacuum into soybean plants (3 to 4 weeks old). Leaves were collected daily for 7 days after infiltration (three leaves at each sample time for each strain). Samples were periodically removed and macerated in 5 ml (final volume) of 0.1 M potassium phosphate buffer and the number of viable cells was determined by dilution plating on LA. The experiments were repeated three times with similar results.

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LITERATURE CITED

- Alonso, J., and Garcia, J. L. 1996. Proline iminopeptidase gene from *Xanthomonas campestris* pv. *citri*. Microbiology 142:2951-2957.
- Arlat, M., Gough, C. L., Zischek, C., Barberis, P. A., Trigalet, A., and Boucher, C. A. 1992. Transcriptional organization and expression of the large *hrp* gene cluster of *Pseudomonas solanacearum*. Mol. Plant-Microbe Interact. 5:187-193.
- Aziz, R. K., Bartels, D., Best, A. A., DeJongh, M., Disz, T., Edwards, R. A., Formisano, K., Gerdes, S., Glass, E. M., Kubal, M., Meyer, F., Olsen, G. J., Olson, R., Osterman, A. L., Overbeek, R. A., McNeil, L. K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G. D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., and Zagnitko, O. 2008. The RAST server: Rapid annotations using subsystems technology. BMC Genomics 9:75.
- Beattie, G. A. 2011. Water relations in the interaction of foliar bacterial pathogens with plants. Annu. Rev. Phytopathol. 49:533-555.
- Bergman, K., Gulashoffee, M., Hovestadt, R. E., Larosiliere, R. C., Ronco, P. G., and Su, L. 1988. Physiology of behavioral mutants of *Rhizobium meliloti*—evidence for a dual chemotaxis pathway. J. Bacteriol. 170:3249-3254.
- Burch, A. Y., Shimada, B. K., Browne, P. J., and Lindow, S. E. 2010. Novel high throughput detection method to assess bacterial surfactant production. Appl. Environ. Microbiol. 76:5363-5372.
- Caetano-Anolles, G., Cristestea, D. K., and Bauer, W. D. 1988. Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. J. Bacteriol. 170:3164-3169.

- Castañeda, A., Reddy, J. D., Yacoubi, B. E., and Gabriel, D. W. 2005. Mutagenesis of all eight *avr* genes in *Xanthomonas campestris* pv. *campestris* had no detected effect on pathogenicity, but one *avr* gene affected race specificity. *Mol. Plant-Microbe Interact.* 18:1306-1317.
- Champoisseau, P., Daugrois, J.-H., Pieretti, I., Cociancich, S., Royer, M., and Rott, P. 2006. High variation in pathogenicity of genetically closely related strains of *Xanthomonas albilineans*, the sugarcane leaf scald pathogen, in Guadeloupe. *Phytopathology* 96:1081-1091.
- Choi, S. H., and Greenberg, E. P. 1991. The C-terminal region of the *Vibrio fischeri* LuxR protein contains an inducer-independent *lux* gene activating domain. *Proc. Natl. Acad. Sci. U.S.A.* 88:11115-11119.
- Cui, Y., Chatterjee, A., Hasegawa, H., Dixit, V., Leigh, N., and Chatterjee, K. 2005. ExpR, a LuxR homolog of *Erwinia carotovora* subspecies *carotovora*, activates transcription of *rsmA* which specifies a global regulatory RNA-binding protein. *J. Bacteriol.* 187:4792-4803.
- Darsonval, A., Darrasse, A., Durand, K., Bureau, C., Cesbron, S., and Jacques, M.-A. 2009. Adhesion and fitness in the bean phyllosphere and transmission to seed of *Xanthomonas fuscans* subsp. *fuscans*. *Mol. Plant-Microbe Interact.* 22:747-757.
- Das, A., Rangaraj, N., and Sonti, R. V. 2009. Multiple adhesin-like functions of *Xanthomonas oryzae* pv. *oryzae* are involved in promoting leaf attachment, entry, and virulence on rice. *Mol. Plant-Microbe Interact.* 22:73-85.
- Datsenko, A. K., and Wanner, B. L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* 97:6640-6645.
- Davey, M. E., and O'Toole, G. A. 2000. Microbial biofilms: From ecology to molecular genetics. *Microbiol. Mol. Biol. Rev.* 64:847-867.
- Ferluga, S., and Venturi, V. 2009. OryR is a LuxR-family protein involved in interkingdom signaling between pathogenic *Xanthomonas oryzae* pv. *oryzae* and rice. *J. Bacteriol.* 191:890-897.
- Ferluga, S., Bigirimana, J., Höfte, M., and Venturi, V. 2007. A LuxR homologue of *Xanthomonas oryzae* pv. *oryzae* is required for optimal rice virulence. *Mol. Plant Pathol.* 8:529-538.
- Fett, W. F., and Dunn, M. F. 1987. Auxin production by plant-pathogenic pseudomonads and xanthomonads. *Appl. Environ. Microbiol.* 53:1839-1845.
- Fett, W. F., Dunn, M. F., Maher, G. T., and Maleeff, B. 1987. Bacteriocins and temperature phage of *Xanthomonas campestris* pv. *glycines*. *Curr. Microbiol.* 16:137-144.
- Foreman-Wykert, A. K., and Miller, J. F. 2003. Hypervirulence and pathogen fitness. *Trends Microbiol.* 11:105-108.
- Fuqua, C., and Greenberg, E. P. 2002. Listening in on bacteria: Acylhomoserine lactone signaling. *Nat. Rev. Mol. Cell Biol.* 3:685-695.
- Galinski, E. A., and Truper, H. G. 1994. Microbial behavior in salt stress ecosystems. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Rev.* 15:95-108.
- Gallegos, M. T., Michán, C., and Ramos, J. L. 1993. The XylS/AraC family of regulators. *Nucleic Acids Res.* 21:807-810.
- Gentleman, R. C., Carey, V. J., Bates, D. M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., Hornik, K., Hothorn, T., Huber, W., Iacus, S., Irizarry, R., Leisch, F., Li, C., Maechler, M., Rossini, A. J., Sawitzki, G., Smith, C., Smyth, G., Tierney, L., Yang, J. Y., and Zhang, J. 2004. Bioconductor: Open software development for computational biology and bioinformatics. *Genome Biol.* 5:R80.
- Hao, G., Zhang, H., Zheng, D., and Burr, T. J. 2005. *luxR* homolog *avhR* in *Agrobacterium vitis* affects the development of a grape-specific necrosis and a tobacco hypersensitive response. *J. Bacteriol.* 187:185-192.
- Hawes, M. C., and Smith, L. Y. 1989. Requirement for chemotaxis in pathogenicity of *Agrobacterium tumefaciens* on roots of soil-grown pea plants. *J. Bacteriol.* 171:5668-5671.
- Hengge, R. 2009. Principles of c-di-GMP signaling in bacteria. *Nat. Rev. Microbiol.* 7:263-273.
- Hokawat, S., and Rudolph, K. 1993. The hosts of *Xanthomonas*. Pages 44-48 in: *Xanthomonas*. J. G. Swings and E. L. Civerolo, eds. Chapman and Hall, London.
- Hugouvieux-Cotte-Pattat, N., Dominguez, H., and Robert-Baudouy, J. 1992. Environmental conditions affect transcription of the pectinase genes of *Erwinia chrysanthemi* 3937. *J. Bacteriol.* 174:7807-7818.
- Hugouvieux-Cotte-Pattat, N., Condemine, G., Nasser, W., and Reverchon, S. 1996. Regulation of pectinolysis in *Erwinia chrysanthemi*. *Annu. Rev. Microbiol.* 50:213-257.
- Huynh, T. V., Dahlbeck, D., and Staskawicz, B. J. 1989. Bacterial blight of soybean: Regulation of a pathogen gene determining host cultivar specificity. *Science* 245:1374-1377.
- Jones, S. B., and Fett, W. F. 1985. Fate of *Xanthomonas campestris* infiltrated into soybean leaves: An ultrastructural study. *Phytopathology* 75:733-741.
- Kaewnum, S., Prathuangwong, S., and Burr, T. J. 2005. Aggressiveness of *Xanthomonas axonopodis* pv. *glycines* isolates to soybean and hypersensitivity responses by other plants. *Plant Pathol.* 54:409-415.
- Kihara, A., Akiyama, Y., and Ito, K. 1996. A protease complex in the *Escherichia coli* plasma membrane: HflKC (HflA) forms a complex with FtsH (HflB), regulating its proteolytic activity against SecY. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:6122-6131.
- Kitten, T., and Willis, D. K. 1996. Suppression of a sensor kinase-dependent phenotype in *Pseudomonas syringae* by ribosomal proteins L35 and L20. *J. Bacteriol.* 178:1548-1555.
- Kovach, E. M., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop II, R. M., and Peterson, K. M. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MSC, carrying different antibiotic-resistance cassettes. *Gene* 166:175-176.
- Landfald, B., and Strom, A. R. 1986. Choline-glycine betaine pathway confers a high level of osmotic tolerance in *Escherichia coli*. *J. Bacteriol.* 165:849-855.
- Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10:R25.
- Le Rudulier, D., Ström, A. R., Dandekar, A. M., Smith, L. T., and Valentine, R. C. 1984. Molecular biology of osmoregulation. *Science* 224:1064-1068.
- Loper, J. E., and Lindow, S. E. 1996. Reporter gene systems useful in evaluating in situ gene expression by soil- and plant-associated bacteria. Pages 482-491 in: *Manual of Environmental Microbiology*. C. J. Hurst, ed. American Society for Microbiology, Washington, DC.
- Malott, R. J., O'Grady, E. P., Toller, J., Inhülsen, S., Eberl, L., and Sokol, P. A. 2009. A *Burkholderia cenocepacia* orphan LuxR homolog is involved in quorum-sensing regulation. *J. Bacteriol.* 191:2447-60.
- Millar, R. K. 1955. Studies on the nature of pathogenicity of *Xanthomonas phaseoli* (E. F. Smith) Downson and *Xanthomonas phaseoli* var. *sojensis* (Hedges) Starr and Burk. Ph.D. thesis, Cornell University. Cited in: *Xanthomonas*. 1993. J. G. Swings and E. L. Civerolo, eds. Chapman and Hall, London.
- Miller, W. G., Leveau, J. H. J., and Lindow, S. E. 2000. Improved *gfp* and *inaZ* broad-host-range promoter-probe vectors. *Mol. Plant-Microbe Interact.* 13:1243-1250.
- Mo, Y. Y., and Gross, D. C. 1991. Plant signal molecules activate the *sydB* gene, which is required for syringomycin production by *Pseudomonas syringae* pv. *syringae*. *J. Bacteriol.* 173:5784-5792.
- Mo, Y. Y., Geibel, M., Bonsall, R. F., and Gross, D. C. 1995. Analysis of sweet cherry (*Prunus avium* L.) leaves for plant signal molecules that activate the *sydB* gene required for synthesis of the phytotoxin, syringomycin, by *Pseudomonas syringae* pv. *syringae*. *Plant Physiol.* 107:603-612.
- Morgan, F. L. 1963. Bacterial pustule of soybean. *Soybean Dig.* 23:8-9.
- Parke, D., Ornston, L. N., and Nester, E. W. 1987. Chemotaxis to plant phenolic inducers of virulence genes is constitutively expressed in the absence of the Ti-plasmid in *Agrobacterium tumefaciens*. *J. Bacteriol.* 169:5336-5338.
- Pinto, U. M., and Winans, S. C. 2009. Dimerization of the quorum-sensing transcription factor TraR enhances resistance to cytoplasmic proteolysis. *Mol. Microbiol.* 73:32-42.
- Poellinger, K. A., Lee, J. P., Parales, J. V., and Greenberg, E. P. 1995. Intragenic suppression of a *luxR* mutation: Characterization of an autoinducer-independent LuxR. *FEMS (Fed. Eur. Microbiol. Soc.) Microbiol. Lett.* 129:97-101.
- Poolman, B., and Glaesker, E. 1998. Regulation of compatible solute accumulation in bacteria. *Mol. Microbiol.* 29:397-407.
- Prathuangwong, S., and Amnuaykit, K. 1987. Studies on tolerance and rate reducing bacterial pustule of soybean cultivars/lines. *Kasetsart J. (Nat. Sci.)* 21:408-420.
- Rahme, L. G., Mindrinos, M. N., and Panopoulos, N. J. 1992. Plant and environmental sensory signals control the expression of *hrp* genes in *Pseudomonas syringae* pv. *phaseolicola*. *J. Bacteriol.* 174:3499-3507.
- Robinson, M. D., McCarthy, D. J., and Smyth, G. K. 2010. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26:139-140.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
- Santiago-Vazquez, L. Z., Ranzer, L. K., and Kerr, R. G. 2006. Comparison of two total RNA extraction protocols using the marine gorgonian coral *Pseudopterogorgia elisabethae* and its symbiont *Symbiodinium* sp. *Electron J. Biotechnol.* 9:598-603.
- Schulte, R., and Bonas, U. 1992. A *Xanthomonas* pathogenicity locus is induced by sucrose and sulfur-containing amino acids. *Plant Cell* 4:79-86.
- Simm, R., Morr, M., Kader, A., Nimtz, M., and Romling, U. 2004. GGDEF and EAL domains inversely regulate cyclic di-GMP levels and transition from sessility to motility. *Mol. Microbiol.* 53:1123-1134.

- Slock, J., VanRiet, D., Kolibachuk, D., and Greenberg, E. P. 1990. Critical regions of the *Vibrio fischeri* LuxR protein defined by mutational analysis. *J. Bacteriol.* 172:3974-3979.
- Stevens, A. M., and Greenberg, E. P. 1997. Quorum sensing in *Vibrio fischeri*: Essential elements for activation of the luminescence genes. *J. Bacteriol.* 179:557-562.
- Stevens, A. M., Dolan, K. M., and Greenberg, E. P. 1994. Synergistic binding of the *Vibrio fischeri* LuxR transcriptional activator domain and RNA polymerase to the *lux* promoter region. *Proc. Natl. Acad. Sci. U.S.A.* 91:12619-12623.
- Subramoni, S., and Venturi, V. 2009. LuxR-family 'solos': Bachelor sensors/regulators of signaling molecules. *Microbiology* 155:1377-1385.
- Subramoni, S., Gonzalez, J. F., Johnson, A., Péchy-Tarr, M., Rochat, L., Paulsen, I., Loper, J. E., Keel, C., and Venturi, V. 2011. Bacterial *subfamily of LuxR regulators that respond* to plant compounds. *Appl. Environ. Microbiol.* 77:4579-4588.
- Tanaka, N., Nagasaka, K., and Komatsu, Y. 2011. RtcB, a novel RNA ligase, can catalyze tRNA splicing and HAC1 mRNA splicing in vivo. *J. Biol. Chem.* 286:30253-30257.
- Tamayo, R., Pratt, J. T., and Camilli, A. 2007. Roles of cyclic diguanylate in the regulation of bacterial pathogenesis. *Annu. Rev. Microbiol.* 61:131-148.
- Thowthampitak, J., Shaffer, B. T., Prathuangwong, S., and Loper, J. E. 2008. Role of *rpfF* in virulence and exoenzyme production of *Xanthomonas axonopodis* pv. *glycines*, the causal agent of bacterial pustule of soybean. *Phytopathology* 98:1252-1260.
- Wang, L., Zhang, L., Geng, Y., Xi, W., Fang, R., and Jia, Y. 2011. XerR, a negative regulator of XccR in *Xanthomonas campestris* pv. *campestris*, relieves its repressor function in planta. *Cell Res.* Published online. doi:10.1038/cr.2011.64
- Weber, C. R., Dunleavy, J. M., and Fehr, W. R. 1966. Effect of bacterial pustule on closely related soybean lines. *Agron. J.* 58:544-545.
- Wei, Z. M., Sneath, B. J., and Beer, S. V. 1992. Expression of *Erwinia amylovora hrp* genes in response to environmental stimuli. *J. Bacteriol.* 174:1875-1882.
- Xiao, Y., Lu, Y., Heu, S., and Hutcheson, S. W. 1992. Organization and environmental regulation of the *Pseudomonas syringae* pv. *syringae* 61 *hrp* cluster. *J. Bacteriol.* 174:1734-1741.
- Zhang, L., Jia, Y., Wang, L., and Fang, R. 2007. A proline iminopeptidase gene upregulated in planta by a LuxR homologue is essential for pathogenicity of *Xanthomonas campestris* pv. *campestris*. *Mol. Microbiol.* 65:121-136.
- Zhu, J., and Winans, S. C. 1999. Autoinducer binding by the quorum-sensing regulator TraR increase affinity for target promoters in vitro and decreases TraR turnover rates in whole cells. *Proc. Natl. Acad. Sci. U.S.A.* 96:4832-4837.