Title

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Journal

Discovery medicine, 12(67)

ISSN

1944-7930

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Publication Date

2011-12-01

Peer reviewed

DISCOVERY MEDICINE®
www.discoverymedicine.com
ISSN: 1539-6509; eISSN: 1944-7930

Small Protein-mediated Quorum Sensing in a Gram-negative Bacterium: Novel Targets for Control of Infectious Disease

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Abstract: Control of Gram-negative bacterial infections of plants and animals remains a major challenge because conventional approaches are often not sufficient to eradicate these infections. One major reason for their persistence seems to be the capability of the bacteria to grow within biofilms that protect them from adverse environmental factors. Quorum sensing (QS) plays an important role in the formation of biofilms. In QS, small molecules serve as signals to recognize bacterial cell population size, leading to changes in expression of specific genes when a signal has accumulated to some threshold concentration. The small protein Ax21 (Activator of XA21-mediated immunity), serves as a QS factor that regulates biofilm formation and virulence in the Gram-negative bacterium, *Xanthomonas oryzae* pv. *oryzae*. Knowledge of small protein-mediated QS in Gram-negative bacteria can be used to develop new methods to control persistent Gram-negative infections. [*Discovery Medicine* 12(67):461-470, December 2011]

Introduction

Biofilms — matrix-enclosed microbial accretions that adhere to biological or non-biological surfaces — are an integral component of the prokaryotic life cycle (Crossman and Dow, 2004; Kaplan, 2010; Murray *et al.*, 2007; Ramey *et al.*, 2004). The elucidation of the molecular mechanisms responsible for the switch from a motile state to a sessile biofilm state and the role of inter-bacterial communication, i.e., quorum sensing (QS), in persistent disease has provided new insights into bacterial pathogenicity (Hall-Stoodley *et al.*, 2004; Verstraeten *et al.*, 2008). We have recently shown that Ax21 (activator of XA21-mediated immunity), a small protein conserved in all species of the Gram-negative bacterial genus *Xanthomonas* and in species of related genera, is critical for virulence and biofilm formation (Han *et al*., 2011).

In this review, we summarize recent research elucidating the molecular basis of Ax21-mediated QS in Gram-negative bacteria and highlight some of important biological activities of this molecule. Our findings have important implications for the development of novel approaches to disrupt Gram-negative bacterial infection.

Plant and Animal Innate Immune Responses Are Triggered by Conserved Microbial Signatures

Innate immunity provides a first line of defense against pathogen attack and is activated rapidly following infection. In contrast to the adaptive immune system that depends on somatic gene rearrangements for the generation of antigen receptors with random specificities, the innate immune system uses a set of defined receptors for pathogen recognition called host immune receptors or pattern recognition receptors (PRR) (Ronald and Beutler, 2010). While it is now widely appreciated that host receptors play a key role in innate immunity in plants and animals, very little is known about the conserved microbial signatures (also called pathogen-associated molecular patterns) recognized by such receptors.

In 1995 we showed that the rice XA21 receptor, encoding a protein with predicted extracellular leucine rich repeat, transmembrane, juxtamembrane, and intracellu-

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lar non-RD (arginine-aspartic acid) kinase domains, confers immunity to strains of the Gram-negative bacterium, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) that express the conserved microbial signature, Ax21 (Lee *et al.*, 2009; Song *et al.*, 1995). Subsequent discoveries in flies (Lemaitre *et al.*, 1996), humans (Kirschning *et al.*, 1998), mice (Poltorak *et al.*, 1998), and *Arabidopsis* (Gomez-Gomez and Boller, 2000; Zipfel *et al.*, 2006) revealed that animals and other plant species also carry receptors with striking structural similarities to the *Xa21*-encoded protein and that these receptors are also involved in microbial recognition and defense. Like XA21, these receptors typically associate with or carry non-RD kinases to control early events of innate immunity signaling (Dardick and Ronald, 2006).

Ax21 Is a Type I-secreted 194 Amino Acid Protein Present in Plant and Animal Pathogens

Based on the broad-spectrum resistance conferred by XA21, and its predicted transmembrane structure, we hypothesized that Xa21 recognized a conserved microbial signature secreted by *Xoo* (Ronald *et al*., 1992*;* Song *et al*., 1995). We used liquid chromatography-mass spectrometry analysis of reverse phase-high pressure liquid chromatography bioactive fractions of *Xoo* strain PX099 to identify such a molecule. We isolated peptides corresponding to a single 194 amino acid protein encoded by a gene that we designated *ax21* (activator of Xa21-mediated immunity*)* (Lee *et al.*, 2009).

The predicted Ax21 protein is present in all sequenced *Xanthomonas* species (90-98% identity), in *Xylella fastidiosa (*48% identity*),* the causal agent of Pierce's disease on grapes, and in the human pathogen, *Stenotrophomonas maltophilia* (61% identity) (Lee *et al.*, 2009). *S. maltophilia* is a Gram-negative bacterium that is widespread in the environment. It has become important in the last 15 years as an emerging opportunistic pathogen associated with nosocomial colonization and infection, such as in cystic fibrosis patients (Ryan *et al.*, 2008).

The Ax21 protein carries two predicted tyrosine sulfation sites. A 17 amino acid synthetic peptide containing sulfated tyrosine-22 ($AxY^{S}22$) is sufficient for Ax21 activity, whereas peptides lacking tyrosine sulfation (AxY22) are inactive. $AxY^{S}22$ binds the XA21 PRR with high affinity (Lee *et al.*, 2009). The 17 aa $AxY^{S}22$ sequence shows 100%, 77%, and 65% identity, respectively, to the corresponding sequences in other sequenced *Xanthomonas* species, to *X. fastidiosa*, and to *S. maltophilia* (Lee *et al*., 2009).

The modification of primary and secondary metabolites by the addition or removal of sulfate can have a profound influence on their biological properties (Bowman and Bertozzi, 1999; Kehoe and Bertozzi, 2000; Mougous *et al.*, 2002). Typically, sulfated molecules are directed outside the cell, where they serve as modulators of cell-cell interactions. A notable example pertinent to agriculture is sulfation of the *Sinorhizobium meliloti* Nod factor (a lipochito-oligosaccharide) that is required for specific recognition by its host alfalfa (Roche *et al.*, 1991). Another example of receptor-ligand reactions controlled by sulfation is the binding of the gp120 subunit of the envelope glycoprotein of the human immunodeficiency virus (HIV) to the human chemokine co-receptors CD4 and CCR5. Sulfation of tyrosine residues in the N-terminal segment of CCR5 appears to be critical for both HIV-1 entry and binding of gp120-CD4 complexes (Farzan *et al.*, 1999; 2000).

Ax21 represents a previously uncharacterized type of conserved microbial signature recognized by a host immune receptor: a small protein. Because XA21 is representative of host receptors controlling innate immunity in other plants and animals, the discovery that Ax21 is a small protein that is a QS factor and is conserved in a human pathogen is expected to have a broad impact on understanding and controlling bacterial diseases of plants and humans (Han *et al*., 2011).

Three Functional Classes of Genes Control Ax21-mediated Cellular Processes

Previous studies, using genetic approaches, led to the identification of ten *Xoo* genes, falling into three functional classes, which are required for activation of XA21-mediated immunity. The first class consists of three genes (*raxA*, *raxB*, and *raxC)* that encode components of a bacterial Type I secretion system (da Silva *et al.*, 2004). RaxB belongs to a subclass of ABC transporters that carry a proteolytic domain that cleaves N-terminal leader sequences before secretion (da Silva *et al*., 2004). The second class includes *raxP* and *raxQ,* which encode an adenosine-5′-triphosphate (ATP) sulfurylase and adenosine-5′-phosphosulfate (APS) kinase, respectively. These proteins function in concert to produce 3′-phosphoadenosine 5′-phosphosulfate (Shen *et al.*, 2002), the universal sulfuryl group donor. This class also includes *raxSt,* which encodes a protein showing similarity with mammalian tyrosyl-sulfotransferases. We have recently shown that *raxSt* encodes a functional sulfotransferase and Ax21 serves as a substrate (Han and Ronald, submitted).

The third class of *rax* genes includes four genes encoding 2 two-component regulatory systems: the RaxR/H system (Burdman *et al.*, 2004) and the PhoP/Q system

(Lee *et al.*, 2008). RaxR/H negatively regulates PhoP, which governs virulence by control of *hrp* gene expression (Lee *et al.*, 2008). The PhoP/Q TCS controls Ax21 expression (Lee *et al.*, 2008). These results suggest that the *Xoo* PhoP/Q TCS functions both in virulence and in the production of Ax21 in partnership with RaxR/H to assess population density. The PhoP/Q TCS confers tolerance to an acidic environment, a condition that *Xoo* likely confronts upon entry into a rice plant. Genetic analysis suggests that RaxH is the bacterial receptor for Ax21 (Han *et al*., 2011). *These results demonstrate the presence of an integrated regulatory circuit that the bacterium utilizes to respond to environmental fluctuations* (Figure 1).

Ax21 Is an Inducer of Density-dependent Gene Expression

In "quorum sensing," small molecules serve as signals to recognize cell population size, leading to changes in expression of specific genes when a signal has accumulated to some threshold concentration (Bassler and Losick, 2006; Bodman *et al.*, 2003; Miller and Bassler, 2001; Fuqua and Winans, 1994). QS signal molecules are involved in bioluminescence, virulence, biofilm formation, sporulation, mating, and competence for DNA uptake, and colonization of new sites (Bassler, 2002; Bassler and Losick, 2006; Fuqua *et al.*, 1996; Lyon and Novick, 2004; Miller *et al.*, 2002; Okada *et al.*, 2005; Taga and Bassler, 2003). Bacteria that are able to signal

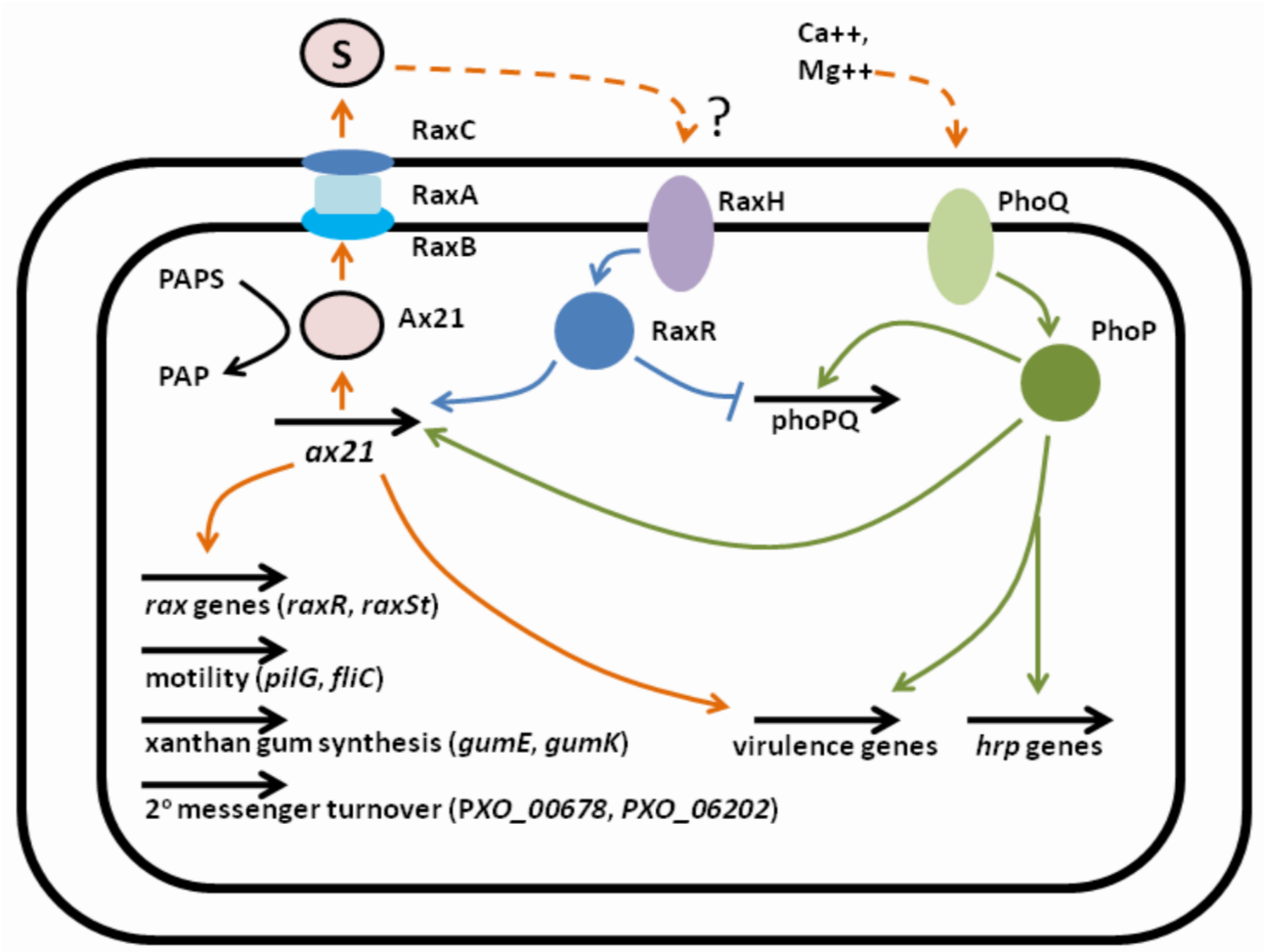

Figure 1. A model for Ax21-mediated quorum sensing. The Ax21 protein is sulfated and transported across the membrane through the RaxABC type I secretion system. The histidine kinase sensor, RaxH, is predicted to bind sulfated Ax21, and activate the response regulator, RaxR, to control gene expression. The histidine kinase PhoQ perceives changes in extracellular ion concentrations and activates the cognate response regulator PhoP to activate Ax21 and virulence gene expression. The interplay between these response regulators controls a switch between motile to sessile modes of growth (Lee *et al*., 2006; Lee *et al*., 2008; Han *et al*., in press, 2011; Han *et al*., submitted; Sriariyanum *et al*., in preparation, 2011b).

to each other and form microcolonies have a competitive advantage in some environments. For example, in *Pseudomonas aeruginosa,* it has been shown that QS has an important role in colonization and virulence in cystic fibrosis, and in most patients it seems that infection is established clonally (Keller and Surette, 2006).

We previously demonstrated that highly purified fractions from *Xoo* carrying Ax21 activity could induce *rax* gene expression at low population density (Lee *et al.*, 2006). These results suggested that Ax21 is involved in QS. We have recently confirmed these results through our demonstration that a purified, recombinant Ax21 protein serves as a QS factor (Han *et al*., 2011). Ax21 plays multiple roles in controlling the establishment of host-microbe interactions: It is required for induction of density-dependent gene expression, motility, virulence, and biofilm formation (Han *et al*., 2011). *To our knowledge this is the first demonstration that a QS factor directly binds a host receptor to trigger innate immunity and that a Gram-negative pathogen uses small protein-mediated quorum sensing to control biofilm formation and virulence.*

One instance of peptide-mediated QS in Gram-negative bacteria has been reported. *E. coli mazEF*-mediated cell death is a population phenomenon requiring a QS molecule called the extracellular death factor (EDF). Unlike the small protein Ax21, EDF is a linear pentapeptide, Asn-Asn-Trp-Asn-Asn (Kolodkin-Gal and Engelberg-Kulka, 2008). *These exciting reports have opened a new and important area of research: How can we use our new knowledge of small protein-mediated QS in Gram-negative bacteria to develop new methods of disease control?*

The Importance of Biofilm Formation in Cell Survival and Virulence

Microbial biofilms are populations of microorganisms that aggregate on a surface (Hall-Stoodley *et al.*, 2004). Biofilm formation represents a protected mode of growth that allows cells to survive in unfavorable environments prior to dispersal and colonization of new niches (Morris and Monier, 2003). A change in behavior from a motile to sessile stage is triggered by many factors, including QS factors, as well as other mechanisms that vary between species. When a cell switches mode to a sessile stage, a number of genes are differentially expressed including genes contributing to exopolysaccharide synthesis such as *gum* genes (Myszka and Czaczyk, 2009; Rigano *et al.*, 2007), pili-dependent bacterial motility (Klausen *et al.*, 2003), and genes controlling cyclic (5′ to 3′)-diguanosine monophosphate (c-di-GMP) synthesis (Ryan *et al.*, 2009). c-di-GMP has emerged as a ubiquitous second messenger in bacteria that controls the transition from a free-living, motile lifestyle to the biofilm lifestyle (Yildiz, 2008). It has also been shown that c-di-GMP turnover controls biofilm formation in many bacterial species such as *Vibrio cholera* (Waters *et al.*, 2008). Higher levels of c-di-GMP cause enhanced biofilm formation. Bacterial cyclic di-nucleotides have also recently been detected in the host cytosol and have been shown to alert the host immune system to the presence of live pathogenic bacteria (Karaolis *et al.*, 2007; McWhirter *et al.*, 2009; Woodward, 2010), highlighting the emerging and important role of these molecules in both virulence and triggering innate immunity.

Work in other laboratories demonstrated that the plant pathogen *Xanthomonas campestris* uses DSF (diffusible signal factor) as a QS factor. DSF is synthesized by the *rpf* (regulation of pathogenicity factors) *F* gene. DSF is perceived by a two-component regulatory system (encoded by *rpfC* and *rpfG*), which controls biosynthesis of the polysaccharide xanthan required for biofilm formation (Slater *et al.*, 2000). The DSF/*rpf* quorum-sensing system also controls production of an extracellular mannanase that is hypothesized to be responsible for dispersal of bacterial aggregates and the return to a motile lifestyle (Dow *et al.*, 2003). Our work demonstrates that *Xanthomonas* carries another, previously unknown QS system mediated by the small protein Ax21 (Han *et al*., 2011).

Ax21 Controls Expression of Genes Involved in Bacterial Motility, Biofilm Formation, and c-di-GMP Metabolism

To identify Ax21-regulated genes, we cultured the *Xoo* PXO99 and PXO99Δax21 strains in PSB (peptone sucrose broth) nutrient-rich media until OD_{600} 0.5, then diluted the cultured cell to a concentration of 10^5 cfu/ml. Cells were then continuously cultured until the cell concentration reached to 10^6 (early log phase), 10^7 (middle log phase), and 10^8 (late log phase) cfu/ml. The same total number of cells from each cell concentration was harvested, and RNA from each sample isolated and hybridized to an *Xanthomonas oryzae* (*Xo*) microarray that contains 4,658 oligonucleotides (50-70-mers) (Seo *et al.*, 2008). Three independent biological replicates of each sample were used. The genes that were differentially expressed more than 1.75 fold (log2ratio >0.8 or <-0.8) and had %FDR (False Discovery Rate) <5% were considered as significant. From this analysis we identified 101 (42 up-regulated and 59 down-regulated genes), 233 (89 up-regulated and 144 down-regulated genes), and 266 (157 up-regulated and 109 down-regu-

lated genes) genes in the 10^6, 10^7, and 10^8 cfu/ml datasets that differ between the PXO99 and PXO99Δax21 strains, respectively. Thus, Ax21 controls expression of ca. 10% of the genome at three different population densities (Han *et al*., 2011).

To elucidate the biological functions of the Ax21-regulated genes, all significant differentially expressed genes were assessed using COG terms (Cluster of Orthologous Groups of proteins). Hierarchical clustering analysis was also applied to assess the pattern of gene expression. During the early log phase nine genes predicted to control bacterial motility [including *fliC*, *fliD*, and *PXO_04752* (chemotaxis gene), are up-regulated 3-, 3-, and 16-fold, respectively] and three genes predicted to control xanthan gum biosynthesis, which have been shown to be important in biofilm formation in *Xanthomonadaceae* (Rigano *et al*., 2007; Souza *et al*., 2006; *gumJ*, *gumE*, and *gumK*, all up-regulated 2-fold) and are up-regulated in PXO99 compared to PXO99Δax21. We also found nine genes encoding proteins that contain HD-GYP, EAL, and GGDEF domains that are up-regulated during the early log phase in PXO99 but not in PXO99Δax21 (from 4- to 30-fold). Proteins carrying these domains were previously demonstrated to be involved in c-di-GMP turnover and shown to trigger a transition from a motile to a sessile stage. In the late log phase dataset, many transcriptional regulators were significantly differentially expressed. For example, the *lexA* repressor and *(ppGpp)ase* genes (ppGpp stands for guanosine-3,5-bis-pyrophosphate) are up-regulated by Ax21. These two genes have previously been shown in *E. coli* to be associated with *rpoS* function [a sigma factor that is expressed during stationary phase and that has been hypothesized to be involved in controlling transition from sessile to motile states (Bougdour and Gottesman, 2007)].

Thus, the observed density-dependent expression of genes regulated by Ax21 indicates precise control of gene expression at different cell densities. In the early log phase, Ax21 up-regulates genes involved in motility and xanthan gum production to form biofilms. In contrast, at later stages (late log phase), many transcriptional regulators are up-regulated. These regulators are hypothesized to be important for regulating stress tolerance and for facilitating a return to the motile stage, which would allow bacteria to break away and seek new sites for infection. Thus, the gene expression analysis supports our hypothesis that Ax21 is a QS molecule that induces gene expression in a cell-density dependent manner (Han *et al*., 2011). We did not observe changes in gene expression of the *rpf* operon in response to Ax21-mediated signaling suggesting that Ax21 likely uniquely contributes to quorum sensing with little overlap with the previously described DSF quorum sensing system (Chatterjee *et al*., 2008; He and Zhang, 2008).

Ax21 Controls Motility Through the Action of the *pilG* and *fliC* Genes

Motility is a group behavior that facilitates bacteria movement on moist surfaces, allowing for rapid colonization of host tissues (Harshey, 2003; Verstraeten *et al*., 2008). Because our microarray data revealed that Ax21 controls genes involved in bacterial motility such as *fliC* and *pilG*, we investigated the effect of Ax21 on motility. We found that the motility of PXO99 was 2-fold higher than that of PXO99Δax21 as measured by diameter of movement in motility assays. Additionally, we made *Xoo* strains carrying knockouts in *fliC* and *pilG* genes and tested their motility. The KO strains had only 66% and 45% motility, respectively, compared to PXO99. These results validate our hypothesis that Ax21 controls motility through the targeted genes and the presence of Ax21 is critical for virulence during early colonization (Han *et al*., 2011).

Ax21 Controls Biofilm Formation Through Action of *gum* Genes

We have shown that Ax21 controls expression of genes predicted to be involved in extracellular polysaccharide (EPS) synthesis such as *gumE* and *gumJ*. We generated strains carrying knockouts in the *gumE* and *gumJ* genes and found that the mutants displayed reduced biofilm formation (about 30%) compared to the wild-type strain. These results validate our hypothesis that Ax21 controls biofilm formation through action of *gumE* and *gumJ*.

Ax21 Controls Biofilm Formation Through Action of Genes Encoding for Protein Involved in c-di-GMP Turnover

We have shown that Ax21 controls expression of nine genes predicted to encode for protein involved in c-di-GMP turnover during early log phase. We generated strains carrying knockouts in *PXO_00678* and *PXO_06202* genes (containing putative GGDEF and EAL domains) and found that the mutants displayed reduced biofilm formation (30% and 20%, respectively) compared to the wild-type strain. These results validate our hypothesis that Ax21 controls biofilm formation through action of c-di-GMP turnover.

Ax21 Is Critical for Virulence at Low Densities

If Ax21 is a QS factor that is critical for virulence, then

a knockout of Ax21 should result in reduced virulence on rice leaves. However, repeated experiments indicate that PXO99Δax21 does not affect virulence using standard inoculation conditions (Kauffman *et al.*, 1973): i.e., clipping leaves with bacteria dipped in high-density cultures 10^8 cfu/ml (Lee *et al.*, 2009). Because under natural conditions, *Xoo* is present at low densities and the minimum concentration of bacterial inoculums required to initiate infection through hydathodes or wounded sites is about 10^4 cfu/ml (Mizukami, 1961), we hypothesized that an effect of Ax21 on virulence may have been masked by the high-density inoculation approach.

To test this hypothesis, we established a new inoculation method with *Xoo* strains PXO99, PXO99*ΔraxSt*, and PXO99Δax21 (Han *et al*., 2011). We found that 90% of the leaves treated with strain PXO99 developed long water-soaked lesions, a typical symptom of the disease. In contrast, only 40% of leaves soaked in low-density cultures of PXO99*ΔraxSt* and PXO99Δax21 developed long lesions. To further quantify these results, we measured bacterial populations. Bacterial populations of strain PXO99 were higher (2-fold) than populations of PXO99Δax21 and PXO99*ΔraxSt* two days following treatment. In contrast, populations of all three strains grew to similar levels using the standard scissors clipping method. These results demonstrate that Ax21 and RaxSt are required for full virulence under conditions that mimic natural field conditions. This experimental data supports field studies, which suggest that spontaneous *Xoo* mutants that lack the ability to secrete Ax21 are impaired in virulence and do not cause epidemics (Choi *et al.*, 2003).

Ax21 Controls Density-dependent Gene Expression and Biofilm Formation in Other *Xanthomonas* Species and Closely Related Genera

Genome sequence analysis indicates that *Xanthomonas axonopodis* pv. *vesicatoria* 85-10 (*Xav*), *X. oryzae* pv. *oryzicola*, *Xylella fastidiosa, Xvm,* and the opportunistic human pathogen *Stenotrophomonas maltophilia* also carry putative Ax21 orthologs (92%, 97%, 48%, 95%, and 61% identity, respectively) (Lee *et al.*, 2009). Because *Xav* carries predicted orthologs for all *rax* genes, including the *raxSt* and *raxA* genes (as does *Xvm*), we hypothesized that *Xav* would express Ax21 activity in the absence of plasmid complementation. Indeed we found that pretreatment of XA21 rice leaves with an *Xav* supernatant triggers XA21-mediated immunity; pretreatment with supernatants from an *XavDax21* strain had no activity (Lee *et al*., 2009). These results indicate that the Ax21 ortholog in *Xav* possesses biological activity. We have also shown that Ax21 regulates density-dependent gene expression in *Xav*. *Xav rax* gene predicted that orthologs (*raxSt*, *raxR*, and *raxA*) are expressed at high cell density in *Xav* but density dependent expression is abolished in the *XavΔax21* strain (Sriariyanun *et al*., 2011a). *Xcc* ATCC 33913 (*Xcc,* non-pathogenic on rice) can express Ax21 activity if the *raxSt* and *raxA* genes from *Xoo* strain PXO99 are provided *in trans* (Lee *et al.*, 2006). A recent report showing that a synthetic Ax21 protein can trigger biofilm formation and control diverse gene expression in *S. maltophilia* confirms the importance of Ax21-mediated activities in a human pathogen (McCarthy *et al*., 2011). These results indicate that the Ax21 sequence conservation in *Xanthomonas* and closely related genera corresponds to functionality.

Conclusions

Despite the recognized importance of bacterial-associated signaling in influencing the outcome of plant and animal diseases, the interactions between these signals and the host are complex and poorly understood. Investigations of the role that small protein-mediated quorum sensing plays in biofilm formation have only just begun. A clear challenge is to determine how quorum sensing works mechanistically to influence virulence. This will require a detailed understanding of small protein-mediated quorum sensing and perception in model organisms and pathogens.

Because 65-80% of human bacterial infections are thought to involve biofilms, including those caused by *Mycobacterium tuberculosis, Staphylococcus aureus*, and *Streptococcus pneumoniae*, understanding the genetic basis of biofilm formation and identifying effective methods to prevent biofilm formation is important for combating disease and for engineering applications (González-Barrios *et al.*, 2006). Chronic infections remain a major challenge for the medical profession and are of great economic relevance because traditional antibiotic therapy is usually not sufficient to eradicate these infections. One major reason for persistence seems to be the capability of the bacteria to grow within biofilms that protects them from adverse environmental factors. In this context, the elucidation of Ax21-mediated biofilm formation has provided new insights into bacterial pathogenicity. This knowledge is expected to lead to the identification of new drug targets for the development of alternative anti-infective treatment strategies that can be used to better manage chronically infected patients (Matz and Kjelleberg, 2005).

While it is now widely appreciated that QS molecules play a key role in bacterial infection of plants and animals, this is the first demonstration of small protein-

mediated quorum sensing in Gram-negative bacteria. To our knowledge, the rice-*Xoo* system is also the first example of type I-secreted small protein that has a dual role in QS and in triggering the host innate immune response. We do not, however, believe this is an anomaly. Given the abundance of predicted QS small proteins in the host vicinity, it will not be surprising if these small proteins are also shown to play a role in the interaction of other bacteria with their hosts. For example, sequence analysis of the genomes of Gram-negative bacteria reveals the abundance of TOSS, two component regulatory systems, and predicted small proteins secreted by these TOSS (Michiels *et al*. 2001). The presence of these systems suggests that the interactions between bacterial pathogens with their environment and their hosts are more sophisticated than currently recognized.

To date, various QS molecules of Gram-negative bacteria, including *Pseudomonas* (Smith and Iglewski, 2003), *Agrobacterium* (Loh et al., 2002), *Vibrio* (Fuqua et al., 1994), and *Xanthomonas* (Barber et al., 1997), have been identified and characterized. QS is an attractive therapeutic target because of the role that it plays in the global regulation of multiple bacterial factors and the importance of its role for the virulence of the organism in multiple different infection stages (Smith, 2003; Crossman and Dow, 2004; Kaplan, 2010; Murray *et al.*, 2007; Ramey *et al.*, 2004; Flemming and Wingender, 2010; González-Barrios *et al.*, 2006). Therefore a "quorum quenching" method has been proposed as an antivirulence strategy that could be added to the existing choices of infection treatment. This approach can prevent pathogenic bacteria from initiating the gene expression cascade required for successful establishment in the host (Persson *et al.*, 2005a). To support this idea, mutant analyses have been performed demonstrating that the lack of quorum sensing correlates with attenuated virulence in both animals and plants (Hussain *et al.*, 2008; Jayaraman and Wood, 2008; Novick and Geisinger, 2008; Quiñones *et al.*, 2005; Han *et al*., 2011).

To date, four approaches have been used to identify quorum sensing inhibitors: (i) chemical synthesis of model compounds based on the natural QS molecule (Rasmussen and Givskov, 2006), (ii) characterization of natural products (Hentzer *et al.*, 2003; Persson *et al.*, 2005b), (iii) screening for natural enzymes involved in degradation of the QS molecule (Chun *et al.*, 2004; Dong *et al.*, 2001), and (iv) screening a large library of synthetic molecules (Borlee *et al.*, 2010; Müh *et al.*, 2006; Swem *et al.*, 2008). Quorum sensing inhibitors identified by these approaches have been shown to disrupt the QS-dependent signal and virulence. For example, one small molecule identified from chemical library screening is a potent antagonist of both LuxN-type membrane bound receptors and LuxR cytoplasmic family of receptors (Swem *et al.*, 2008). The most potent antagonist protects *Caenorhabditis elegans* from quorum-sensing-mediated killing by *Chromobacterium violaceum*, validating the notion that targeting quorum sensing has potential for disease control (Swem *et al.*, 2009). These studies make a strong case and provide compelling *in vivo* evidence that an anti-quorum-sensing strategy is a valid alternative for disease control of Gram-negative bacteria. However, to our knowledge, all of these studies focus on AHL-mediated QS, none have been developed and tested for other QS systems.

Thus, studies of Ax21 have advanced the conceptual framework underlying our understanding of Gram-negative bacterial infection and will lead to new interventions that will drive strategies to control nocosomial infections and crop disease. Such novel QS factors will likely serve as new drug targets to control deadly groups of bacteria, for which there are currently no effective treatments (Boucher *et al.*, 2009; Tripathi *et al.*, 2009).

Acknowledgments

I am grateful for informative discussions with Malinee Sriaryanum, Sang Won Lee, and Sang-Wook Han. This work was supported by the National Institutes of Health grant R01 GM59962 and USDA grants 2006-01888 and 2007-35319-18397 to P.C.R.

Disclosure

The author has no financial conflicts of interest to disclose.

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