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Secretion, modification, and regulation of Ax21

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Innate immunity provides a first line of defense against pathogen attack and is activated rapidly following infection. Although it is now widely appreciated that host receptors of conserved microbial signatures play a key role in innate immunity in plants and animals, very little is known about the biological function of the microbially derived molecules recognized by such receptors. We have recently demonstrated that the rice XA21 receptor binds the AxY^{S}22 peptide corresponding to the N-terminal region of Ax21, a type I-secreted protein that is highly conserved in all *Xanthomonas* species as well as in *Xylella fastidiosa* and the human pathogen, *Stenotrophomonas maltophilia*. We hypothesize that post-translational modification of Ax21 is carried out by the RaxP, RaxQ, and RaxST proteins and that perception and regulation of Ax21 is controlled by the RaxR/H and PhoP/Q 2-component regulatory systems. Ax21 is predicted to serve as an inducer of quorum sensing (QS), a process where bacteria communicate with one another. Because this is the first example of a conserved microbial signature that binds a host receptor and is also predicted to serve as an inducer of QS, this work has revealed fundamental new principles governing host-microbe interactions and has provided insight into the signaling dynamics of microbial communities.

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Introduction

Innate immunity in animals and plants, provides a first line of defense against diverse pathogens 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 in animals, the innate immune system uses a set of defined receptors for pathogen recognition called host sensors or pattern recognition receptors (PRRs) [1].

In 1995 we showed that the rice *Xa21* gene, which encodes a protein with predicted extracellular leucine-rich repeat (LRR), transmembrane, juxtamembrane, and intracellular kinase domains, confers immunity to the Gram-negative bacterium *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) [2••,3]. Subsequent discoveries in flies [4], humans [5], mice [6], and *Arabidopsis* [7,8] revealed the presence of proteins with structures strikingly similar to XA21. These proteins were also shown to be involved in microbial recognition and defense. Like XA21, these receptors typically associate with or contain non-RD (arginine–aspartic acid) kinases to control early events of innate immunity signaling [9]. We have recently shown that XA21 recognizes a conserved microbial signature, termed Ax21 (activator of XA21-mediated immunity) [2••].

Other conserved microbial signatures [also called PAMPs (pathogen associated molecular patterns)] recognized by plant and animal receptors include flagellin, a proteinaceous component of bacterial flagella (recognized by human TLR5 and *Arabidopsis* flagellin-sensitive 2 (FLS2); [7,10]), lipopolysaccharide of Gram-negative bacteria (recognized by TLR4; [11]), the elongation factor-Tu (recognized by elongation factor Tu receptor (EFR), [8]), and peptidoglycan of Gram-positive bacteria [12] (see Segonzac and Zipfel, this issue). For some conserved microbial signatures, post-translational modifications such as glycosylation (*Pseudomonas aeurginosa*) or acylation (*Yersina pestis*) can affect the specificity of host recognition [13–15].

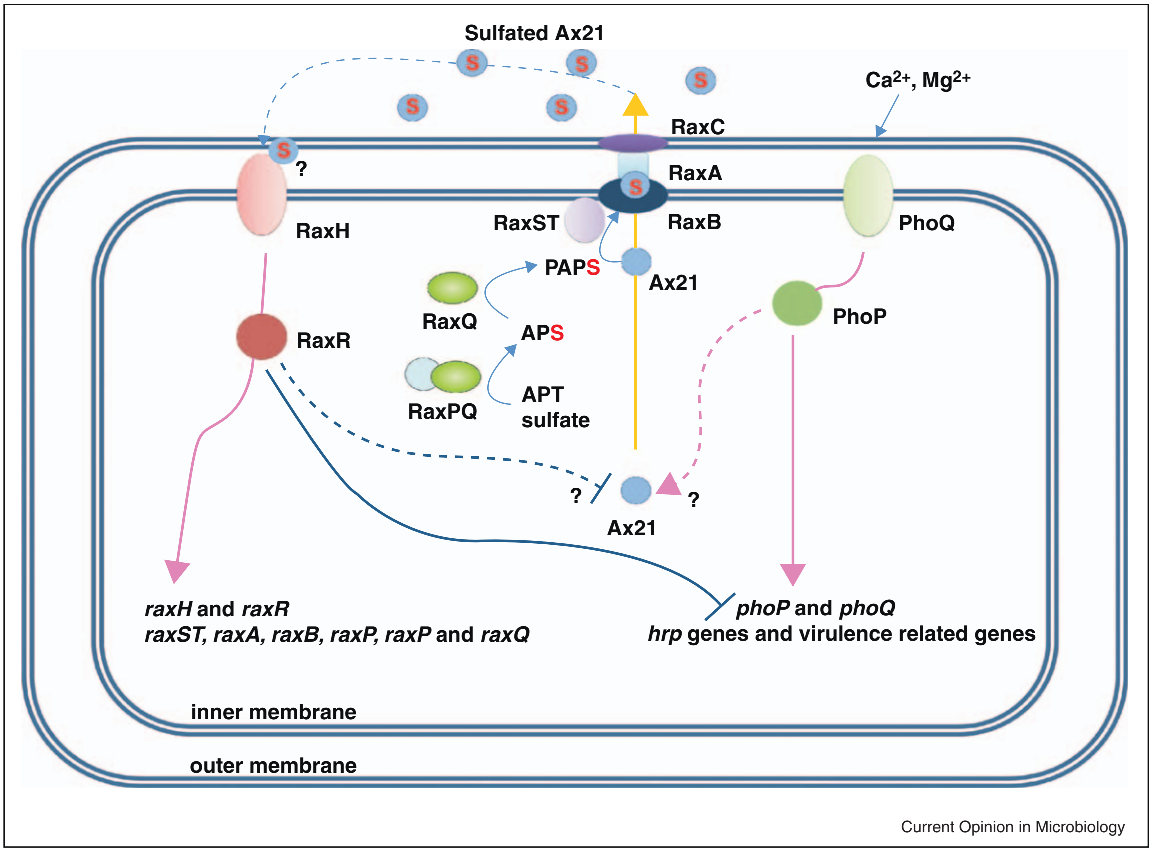
Given the demonstrated importance of host receptors in innate immunity, there is great interest in elucidating the biological function of conserved microbial signatures that they detect [9].

Ax21 is a type I-secreted protein present in plant and animal pathogens

Type I secretion systems (TOSS), also known as the adenosine triphosphate binding cassette (ABC) transporter dependent pathway, is one of the most well-characterized secretion systems in bacteria. TOSS consist of only three protein subunits: the ABC protein, a membrane fusion protein (MFP), and an outer membrane protein (OMP) [16]. The TOSS are involved in the transport of β-glucans, polysaccharides, and toxins [17–19]. In addition, small peptides and proteins in various sizes are secreted via TOSS [20,21].

We have recently shown that *ax21* from *Xoo* encodes a sulfated protein secreted by the TOSS (Figure 1)

Figure 1



Model for Ax21-mediated quorum sensing. Ax21 is sulfated by RaxST, which is a putative tyrosine sulfotransferase. RaxST utilizes 3′-phosphoadenosine 5′-phosphosulfate (PAPS), the production of which is catalyzed by RaxP, an adenosine-5′-triphosphate (ATP) sulfurylase, and RaxQ, adenosine-5′-phosphosulfate (APS) kinase. Sulfated Ax21 is secreted via the RaxABC type I secretion system. Sulfated, secreted Ax21 is recognized by the rice XA21 receptor. The PhoP/Q two-component regulatory system can sense low concentrations of Mg^{2+} and Ca^{2+} ions present in the environment which triggers expression of *hrp* and virulence-related genes. Bacteria then multiply, resulting in accumulation of Ax21, which is sensed by RaxR/RaxH. This in turn leads to upregulation of *rax* genes and repression of *hrp* and *phoP* genes. Thus, we hypothesize that the two two-component regulatory systems control different stages of bacterial growth and infection as described in the text. In this model, Ax21 serves as a quorum sensing factor. S (red color) indicates addition of a sulfuryl group.

[2,22,23]. We carried out liquid chromatography–mass spectrometry analysis of reverse phase-high pressure liquid chromatography bioactive fractions of supernatants from *Xoo* strain PXO99. This work led to the identification of peptides corresponding to a single 194 amino acid protein encoded by *ax21* [2••]. An *Xoo* mutant strain lacking *ax21* (PXO99*Δax21*) is no longer recognized by XA21 rice lines. *Xoo* mutants defective in the TOSS no longer secrete Ax21 and lose the ability to trigger XA21-mediated resistance.

Ax21 is present in all sequenced *Xanthomonas* species (90–98% amino acid sequence identity), in *Xylella fastidiosa* (48% identity), the causal agent of Pierce's disease on grapes, and in the human pathogen *Stenotrophomonas maltophilia* (61% identity) [2••]. *S. maltophilia* is a Gram-negative bacterium that is widespread in the environment and that has become important in the last 15 years because it is an emerging opportunistic pathogen associated with nosocomial colonization and infection [2••,24•].

Modification of Ax21

Tyrosine sulfation is one of the most abundant post-translational modifications [25]. In contrast to phosphorylation, which regulates processes that occur inside the cell, sulfated proteins/peptides are typically directed to the outside of the cell where they modulate cell–cell interactions and ligand–receptor interactions.

A notable example pertinent to agriculture is sulfation of the *Sinorhizobium meliloti* Nod factor that is required for specific recognition by its host alfalfa [26]. In humans, sulfation of residues in the C-terminus of the alpha subunit of the hCG (human glycoprotein choriogonadotropin) ligand is required for binding with the N-terminal LRR domain of the hCG receptor [27,28]. Another example of regulation 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 [29,30].

We have shown that a 17 amino acid synthetic peptide containing a sulfated tyrosine-22 (AxY^S22), derived from the N-terminal region of Ax21, is sufficient for triggering XA21-mediated immunity. In contrast, peptides lacking tyrosine sulfation are inactive [2••]. We have shown that the AxY^S22 peptide binds the XA21 receptor [2••]. The AxY^S22 N terminal peptide is 100% conserved in all *Xanthomonas* species. *X. fastidiosa* and *S. maltophilia* show 77% and 65% identity to the AxY^S22 N terminal peptide sequence, respectively [2••]. Thus, AxY^S22 represents a previously uncharacterized type of conserved microbial signature recognized by host receptors: a sulfated peptide. Our studies suggest that *Xoo* evades XA21-mediated recognition by altering secretion and/or post-translational modification of the Ax21 protein. Because the rice XA21 protein is representative of receptors controlling innate immunity in plants and animals, the discovery that AxY^S22 is a sulfated peptide and that it is conserved in a human pathogen is expected to have a broad impact on understanding and controlling bacterial diseases of plants and humans.

Genes required for activation of XA21-mediated immunity (*rax* genes)

We identified three genes, *raxA*, *raxB* and *raxC*, which encode MFP, ABC, and OMP, respectively, that are required for the secretion of Ax21 (hence *rax* genes). In addition, we demonstrated that *raxP* and *raxQ*, which encode an adenosine-5′-triphosphate sulfurylase and adenosine-5′-phosphosulfate kinase, respectively are also required for Ax21 activity. These proteins function in concert to produce 3′-phosphoadenosine 5′-phosphosulfate (PAPS) [31], the universal sulfuryl group donor. The *raxST* gene encodes a protein with similarity to mammalian tyrosine-sulfotransferases. We hypothesize that RaxST catalyzes the transfer of the sulfuryl-group from PAPS to the tyrosine residue(s) of Ax21 (Figure 1).

Furthermore, two different two-component regulatory systems (TCSs) are required for Ax21 activity: the RaxR/H system [32] and the PhoP/Q system [33]. RaxR shows 44% amino acid similarity to response regulators (RRs) of the well-studied *E. coli* OmpR subfamily. Similarly to OmpR, RaxR is composed of an N-terminal response regulator (receiver) domain and a C-terminal transcriptional regulator (effector) domain. RaxH belongs to the Histidine Protein Kinase 2 subfamily of Histidine Kinases (HKs), which contains ColS and EnvZ. SMART (Simple Molecular Architecture Research Tool in ExPASy proteomics server, http://smart.embl-heidelberg.de/) search also revealed the presence of two transmembrane segments, suggesting that RaxH is a typical transmembrane HK. RaxR is a substrate for RaxH trans-phosphorylation [32]. We demonstrated that the *Xoo phoP* gene, which encodes a response regulator, is up-regulated in the *raxR* knockout (PXO99Δ*raxR*) strain [33].

Ax21 is predicted to be an inducer of density-dependent gene expression

In quorum sensing (QS), small molecules (QS factors), often called ‘auto-inducers’, serve as signals that are recognized in a population-dependent manner. When the signal accumulates to a particular threshold concentration, massive changes in gene expression are triggered [34]. QS signal molecules are involved in motility, adhesion, virulence, biofilm formation, sporulation, mating, and competence for DNA uptake in both Gram-positive and Gram-negative bacteria [35•,36–41]. QS is also hypothesized to function in the colonization of a new site. Bacteria that are able to signal to each other and form microcolonies have a competitive advantage in some environments. For example, in *Pseudomonas aeruginosa*, QS is involved in colonization and virulence in cystic fibrosis patients [42].

We have shown that highly purified fractions carrying Ax21 activity are required for the induction of density-dependent *rax* gene expression in wild-type *Xoo* strains [23]. A strain lacking RaxR (PXO99Δ*raxR*) no longer expresses *rax* genes at high density. These results suggest that RaxH and RaxR serve as receptor and response regulator, respectively, for Ax21-mediated QS [23]. Experiments are underway to purify the Ax21 protein from *Xoo* and to test if the isolated protein is sufficient to serve as a QS molecule. We will also test if purified Ax21 directly binds RaxH. Although a QS molecule from *P. aeruginosa* has been shown to stimulate phagocytic activity in human macrophages through a MAPK pathway, it is not known if QS molecules directly bind to host immune receptors [43,44].

To date, there is only one instance of peptide-mediated QS in Gram-negative bacteria. *mazEF* is a toxin-antitoxin operon present in many bacterial chromosomes, including pathogens [45••]. *E. coli mazEF*-mediated cell death requires a QS molecule termed extracellular death factor (EDF). Structural analysis revealed that EDF is a linear pentapeptide, Asn-Asn-Trp-Asn-Asn. Each of the five amino acids of EDF is important for its activity. The cellular component(s) directly interacting with EDF and the specific stage(s) affected in the *mazEF*-mediated death network are not known yet.

The PhoPQ two-component regulatory system detects and responds to extracellular nutrient status and controls expression of *hrp* genes

Pathogens have evolved integrated regulatory circuits that control the coordinated expression of one set of genes in one environment and a different set of genes in another. In pathogenic bacteria, these regulatory circuits are generally controlled by TCSs, composed of HKs and RRs. In response to environmental stimuli, the HKs phosphorylate the cognate RRs, which then activate gene expression [46].

In *Salmonella typhimurium*, PhoQ activity is modulated by extracellular levels of Mg^{2+} and Ca^{2+}. Low cation concentrations promote activation of *mgtA*, *pbgC*, *pcgF*, *pcgG*, *mgtCB*, and *psiD* genes whereas high concentrations result in the repression of these genes [47,48]. These results indicate that *Salmonella* PhoQ is a sensor for periplasmic concentrations of divalent cations. The role of divalent cations as signals for PhoQ is also supported by the crystal structures of the PhoQ periplasmic sensor domains from *S. typhimurium* and *E. coli* [48]. Similarly, we have shown that the *Xoo* PhoPQ system is required for sensing low extracellular Mg^{2+} and Ca^{2+} concentrations, conditions that the pathogen likely is confronted with upon entry into the xylem of the rice plant [33]. In addition, we have shown that Ax21 activity is impaired in a *phoQ* knockout strain (PXO99*ΔphoQ*) as reflected by enhanced growth of this strain in rice lines carrying XA21 [33]. These data suggest that PhoQ not only senses divalent cations but also regulates Ax21 activity.

Which biological activities, then, are controlled by the PhoP/Q regulatory system? We have reported that a *phoP* knockout strain (PXO99*ΔphoP*) is impaired in *Xoo* virulence and is no longer able to activate the response regulator HrpG (hypersensitive reaction and pathogenicity G) in response to low levels of Ca^{2+}[33]. The impaired virulence of the PXO99*ΔphoP* strain can be partially complemented by constitutive expression of *hrpG*, indicating that PhoP/Q controls a key aspect of *Xoo* virulence through regulation of *hrpG*. These results are reminiscent of the fact that the PhoP/Q TCS is required for virulence in *Shigella flexneri* and *Yersinia pestis* [49,50].

In *Xanthomonas* spp. and *R. solanacearum*, HrpG activates *hrpX* and *hrpA* expression. In turn, HrpX upregulates the expression of the *hrpB* to *hrpF* operons, which encode components of a type III secretion system (T3SS). The T3SS secretes proteins directly into host cells. HrpX also controls expression of type III effectors (T3E), which are secreted proteins via the T3SS [51,52]. We have shown that expression of *hrpA* and *hrpX* in *Xoo* is significantly higher in the presence of low Ca^{2+} concentrations in the wild-type strain, but not in the PXO99*ΔphoP* strain [33]. These results demonstrate that the PhoP/Q TCS senses cation concentrations to regulate *hrp* gene expression through HrpG.

Because we have shown that RaxR negatively regulates *phoP* gene expression [33], we hypothesized that *hrpG*, *hrpA*, and *hrpX*, which are positively regulated by PhoP, would be negatively regulated by RaxR. Indeed, we found that 23 *hrp* and *hrp*-related genes, including *hrpG*, *hrpA*, and *hrpX*, are down-regulated in RaxR overexpression strains and up-regulated in the PXO99*ΔraxR* strain [33]. These results support a model in which *Xoo hrp* gene expression is under control of PhoP, which in turn is negatively regulated by the RaxR/H TCS (Figure 1).

According to this model, the *Xoo* PhoP/Q TCS works in partnership with RaxR/H to assess population density and to control regulation of effectors. Our results suggest the presence of an integrated regulatory circuit that the bacterium utilizes to respond to environmental fluctuations (Figure 1).

Conclusion

In summary, Ax21 from *Xoo* is a secreted, sulfated protein that is widely conserved in *Xanthomonas* and closely related genera. We hypothesize that the biological function of Ax21 is as a QS signal molecule, perception of which controls the production of diverse cellular processes in a cell-density-dependent manner via the *Xoo* RaxR/H and/or PhoP/Q TCSs.

These findings lead to the hypothesis that Ax21 triggers a transition from a quiescent or epiphytic state to an invasive or pathogenic state of the bacterium in response to changing extracellular conditions sensed by the two TCSs. This hypothesis would explain why the PhoP/Q TCS, which triggers expression of a set of genes, including *hrp* genes, through the negative regulation of RaxR/H, is also required for Ax21 activity. Because *Xoo* must monitor population size under changing conditions, an integrated and flexible response system is desirable. In this model (Figure 1), *Xoo* can sense low concentrations of Mg^{2+} or Ca^{2+} in the host via the PhoP/Q TCS. These conditions would trigger *phoP*-regulated gene expression. The consequence would be an increased expression of genes required for virulence such as *hrp* genes. Bacteria would then propagate, resulting in the accumulation of Ax21, which is sensed by the RaxR/H TCS. This perception would lead to upregulation of *rax* genes and repression of *hrp* genes.

The discovery of Ax21 and its regulatory system is a fascinating first step for understanding the role of Ax21 in the bacterial lifecycle and its interaction with its host. There are still many questions that remain to be answered about the role of Ax21 and its regulation. For example (1) is Ax21 a QS factor? (2) Which kind of genes are controlled by Ax21-mediated QS in *Xoo* and what are their functions? (3) Are there any other components required for activation and regulation of Ax21? In addition to broadening our knowledge of the bacterial disease process, the study of the integrated circuitry system controlled by Ax21 may lead to the development of new strategies of disease control in plants and animals.

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