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Biosynthesis of diffusible signal factor (DSF) signals in *Xanthomonas campestris* pv. *campestris* is induced by host metabolites

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Quorum sensing (QS) denotes a widely conserved cell-to-cell communication mechanism which coordinates bacterial group behavior and often regulates virulence, biofilm formation, antibiotic production and plasmid conjugal transfer. The cell-cell communication signal cis-11-methyl-2-dodecenoic acid (also known as DSF) was originally identified in *Xanthomonas campestris* pv. *campestris* (Xcc), representing a widely conserved signaling mechanism in many Gram-negative bacterial pathogens. The signal is involved in the regulation of biofilm dispersal and virulence. Previous work showed that DSF biosynthesis in Xcc is dependent on RpfF and RpfB, but it is not clear how host may affect its production. Here we report that exogenous addition of the cell-free extract from Chinese cabbage to the growth medium of Xcc significantly induces the DSF-family signal production. The further study showed that the biosynthesis of BDSF and DSF are significantly enhanced. Our works reveal that Xcc can utilize host metabolites in Chinese cabbage to increase quorum sensing signal production, and facilitate its infection.

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Functional characterization of genes encoding HD-GYP domain proteins in *Xanthomonas oryzae* pv *oryzicola*

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Bacterial leaf streak caused by *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) is one of important diseases in rice. However, little is known about the pathogenicity mechanisms of *Xoc*. Here, we investigated the function of the three Xoc HD-GYP proteins including RpfG in biofilm formation, the production of extracellular polysaccharides, the secretion of extracellular enzymes, as well as virulence on rice. Deletion of *rpfG* resulted in decreased production of extracellular polysaccharides, abolished *Xoc* virulence on rice, but enhanced biofilm formation. Biochemical studies including colorimetric assays, HPLC and mass spectrometry demonstrated that RpfG is a phosphodiesterase that hydrolyses c-di-GMP into GMP via linear pGpG as an intermediate degradation product. Cross-complementation of the *Xoc rpfG* mutant with *rpfG* from *X. campestris* (*Xcc*) restored the mutant phenotypes, but *Xoc rpfG* did not cross-complement the *Xcc rpfG* mutant in EPS production and the secretion of extracellular enzymes. Expression analysis showed that deletion of *rpfG* significantly increased expression of the type III secretion system (T3SS) and *pgaABCD* operon that is required for biofilm formation in *Escherichia coli*. The other two HD-GYP domain proteins have no effect on virulence factor synthesis and tested phenotypes. The results indicated that RpfG in *Xoc* positively controls the production of extracellular polysaccharides and virulence on rice, but negatively regulates biofilm formation and T3SS expression. The results also suggested that the *pgaABCD* operon is likely involved in biofilm production in *Xoc*.

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Interactions of HrpB proteins in *Xanthomonas oryzae* pathovar *oryzae*

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Xanthomonas oryzae pathovar *oryzae* is the causal agent of rice bacterial blight disease. The type III secretion system of *X. oryzae* pathovar *oryzae*, encoded by *hrp* (hypersensitive response and pathogenicity) gene cluster, is necessary for both pathogenicity in susceptible hosts and the induction of the hypersensitive response in resistant. In this cluster, we were focusing at the function of HrpB proteins encoded by the *hrpB* operon - HrpB1, HrpB2, HrcJ, HrpB4, HrpB5, HrcN, HrpB7 and HrcT, which were not well characterized except HrcN as ATPase. We hypothesized that these HrpB proteins may work together, so we tested the interactions among these eight proteins by yeast two-hybrid. We cloned these eight *hrpB* genes to bait vector-pGBKT7 and prey vector-pGADT7. We carried out co-transformation with these eight baits and eight preys to yeast strain AH109 and 64 combinatorial tranformants were formed. Sixty-four yeast transformants were tested about livability on the auxotroph medium and blue color on the x-α-gal plate. As a result, HrpB2, HrpB4, HrpB5, HrcN and HrpB7 proteins have interactions among them, but, HrpB1, HrcJ and HrcT proteins have no interactions. Specially, in case HrpB2, HrpB5, HrcN and HrpB7 proteins showed self-interactions. So we suggest that the proteins encoded by *hrpB* operon work together to their plant pathogenic function.

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Substrate specificity switching during type III secretion in the plant pathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria*

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The Gram-negative bacterial plant pathogen *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) utilizes a type III secretion (T3S) system to translocate a large set of bacterial effector proteins directly into the eukaryotic cell. The T3S system is a highly complex nanomachine that spans both bacterial membranes and is associated with an extracellular pilus and a predicted translocon in the plant plasma membrane. Given the architecture of the T3S system, it is assumed that pilus and translocon formation precedes effector protein translocation and that there is a switch in the T3S substrate specificity from early to late substrates. T3S substrate specificity switching in *Xcv* presumably depends on the switch protein HpaC and the cytoplasmic domain of the inner membrane protein HrcU ($HrcU_C$), which is autoproteolytically cleaved and is presumably involved in the recognition of secreted proteins. $HrcU_C$ interacts with HpaC and the early T3S substrate HrpB2, which is required for pilus assembly and probably functions as a periplasmic component of the T3S system at the base of the pilus. A predicted conformational change in $HrcU_C$ that presumably occurs after the binding of HpaC leads to the substrate specificity switch after pilus formation. Interestingly, the results of mutant and interaction studies suggest that HrpB2 and HpaC compete for the same binding site in HrcU and that the secretion of early and late substrates is controlled by different mechanisms that can be uncoupled.

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Variations in type III effector repertoires do not correlate with differences in pathological phenotypes and host range observed for *Xanthomonas citri* pv. *citri* pathotypes

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Xanthomonas citri pv. *citri* (Xac) is a quarantine bacterium causing Asiatic citrus canker. Strains of Xac are classified as pathogenic variants i.e. pathotypes, according to their host range: strains of pathotype A infect a wide range of rutaceous species, whereas strains of pathotype A*/A^w infect a restricted host range consisting of Mexican lime (*C. aurantifolia*) and alemow (*C. macrophylla*). Based on a collection of 55 strains we investigated the role of type III effectors (T3E) in host specialization. By PCR we screened 56 *Xanthomonas* T3Es and showed that Xac possesses a repertoire of 28 effectors, 24 of which are shared by all strains, while 4 (*xopAI, xopAD, xopAG* and *xopC1*) are present only in some A*/A^w strains. However, their distribution could not account for host specialization. *XopAG* is present in all A^w strains, but also in three A* strains genetically distant from A^w , and all *xopAG*-containing strains induced HR-like reactions on grapefruit and sweet orange. A strains are genetically less diverse, induce identical phenotypic responses, and share exactly the same T3Es. Conversely, A*/A^w strains exhibited a wider genetic diversity in which clades correlated to geographical origin and T3Es repertoire but not to pathogenicity. A*/A^w strains showed a broad range of reactions on several *Citrus*, but genetically related strains did not share phenotypic responses. Our results showed that A*/A^w strains are more variable (genetically and pathogenetically) than initially expected and that this variability should not be ignored when trying to describe mechanisms involved in the pathogen evolution and host specialization.

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Regulons of *expA* and *rsmA* in *Pectobacterium* strain SCC3193

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Two regulatory genes; *rsmA* and *expA* are well distributed among many enterobacteria and have been extensively studied to date. These two genes have been shown to have an impact on functions such as metabolism, motility and virulence. They have also been linked to the same regulatory pathway when concerning the production of plant cell-wall degrading enzymes (PCWDEs), with *expA* controlling *rsmA* expression through genes such as *rsmB*. The genome of the *Pectobacterium* strain SCC3193, was recently sequenced. Previous work with this strain has revealed that knock-out mutants in *expA* exhibit highly reduced virulence on plants, along with reduced production of PCWDEs. In contrast, knock-out mutants of *rsmA* in SCC3193 demonstrate increased production of PCWDEs, and increased expression of many virulence related genes. Since previous studies indicate that *expA* and *rsmA* operate in the same pathway of regulating the production of PCWDEs, we wanted to further explore the overlap of their regulons and to determine if *expA* exerts its influence through *rsmA* in other aspects of bacterial physiology. We have thus conducted gene expression microarray experiments to determine the transcriptome of knock-out mutants in *expA* and *rsmA*, as well as a double knock-out mutant. Our work reveals synergies and divergence of the transcriptomes of these two genes involved in global genetic regulation, with impact on genes directly and indirectly involved in virulence. In addition to the microarray we have performed assays of growth, virulence and enzyme production, linking the transcriptomic differences of the mutants to phenotypic differences in virulence.

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Quorum sensing mechanism mediates virulence control in the plant pathogen *Xylella fastidiosa*

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The xylem-limited plant pathogen *Xylella fastidiosa*, that colonizes the grape vascular system employs a Diffusible Signal Factor (DSF) to control virulence. DSF is synthesized by RpfF and sensed by the RpfCG phosphorelay system that modulates cyclic di-GMP metabolism that serves as a switch to transition between a motile plant-colonizing phase and a more adhesive, non-motile form that can be vectored by insect vectors. *rpfF* and *rpfG* mutants migrate faster in the plant, proliferate more, cause more symptoms, are less "sticky" than the wild type strain, but are not transmissible; both mutants exhibit lower expression of traits contributing to biofilm formation such as hemagglutinin-like proteins including HxfA and higher expression of genes associated with motility, growth and proliferation. DSF consists of one or more unsaturated fatty acids including 2-Z-tetradecanoic acid DSF; it is active at concentrations as low as 1 μM as measured using *hxfA::phoA* transcriptional fusions in *X. fastidiosa*. In addition, adhesiveness of *X. fastidiosa* increased while growth was suppressed in response to exogenous synthetic 2-Z-tetradecanoic acid. We propose that DSF anti-virulence activity may have evolved to avoid excessive colonization of xylem vessels that is lethal to *X. fastidiosa*. Disease control can be achieved in a process of pathogen confusion in which DSF levels are elevated in plants in advance of pathogen infection by topical application and by expression of *rpfF* in transgenic grape.

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Identifying factors involved in pathogenicity of *Ralstonia solanacearum* strains at low temperatures using a proteomics approach

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Ralstonia solanacearum species is well adapted to life in subtropical and tropical regions thus most of the populations are non-pathogenic below 20^{o}C, however R3B2 strains not established in the U.S. have been identified to cause disease at low temperatures. Due to risks associated with cool virulent strains, knowledge regarding pathogenicity at low temperatures is needed to facilitate effective disease control. In order to identify putative proteins/pathways possibly involved in pathogenicity at low temperatures, we compared protein levels of two strains of *R. solanacearum* that are not naturally pathogenic at low temperatures (P597, GMI1000) and two strains that are cool virulent (P673, UW551) at 30^{o}C and 18^{o}C. Proteins were extracted and 2-D DIGE protein gels were run in several experiments. Comparisons were made for cellular and secreted proteins when *R. solanacearum* cells were incubated in co-culture with tomato seedlings grown *in vitro* in liquid medium, focusing our attention to the root colonization phase of disease progress. The differential profiles of various comparisons in 5 experiments produced 164 proteins mostly involved with survival in a hostile environment. After exhaustive analysis 29 unique proteins were identified as best potential candidates for cold virulence factors. The candidates include a catalase, PilQ, exoglucanase A, a drug efflux pump, and two hypothetical proteins. Currently we are confirming differential regulation of selected candidates using qRT-PCR and testing their putative role in virulence at low temperature. Preliminary experiments suggest that virulence at 30^{o}C of P673 PilQ defective mutants is not significantly reduced; however it is at 18^{o}C.