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Comparative proteomics reveal new HrpX-regulated proteins of *Xanthomonas oryzae* pv. *oryzae*☆

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ABSTRACT

Pathogenicity of the rice pathogenic bacterium *Xanthomonas oryzae* pv. *oryzae* depends on a Hrp (hypersensitive response and pathogenicity) type III secretion system; the expression of which is induced in planta. Expression of the *hrp* operons is under transcriptional control of two key regulatory proteins, HrpG and HrpX. To identify new proteins that are co-regulated with the type III secretion system, we employed comparative proteomics. Cells of *X. oryzae* pv. *oryzae* ectopically expressing *hrpX* were compared to wild-type cells grown *in vitro*. Twenty protein spots with different abundances in both samples were identified by 2D-DIGE and LC–MS/MS. Seven spots could be unambiguously identified, corresponding to the HrpB1 protein, two different peptidyl–prolyl *cis-trans* isomerases, a component of an ATP binding cassette (ABC) transport system, an adenylate kinase, and a secreted protein of unknown function. Interestingly, the isoelectric point of the adenylate kinase was found to be under control of HrpX, most likely due to post-translational modification. Indeed, two glutamate residues of the adenylate kinase were found to be methylated but this modification did not account for the shift in electrophoretic mobility. In summary, we identified new HrpX-regulated proteins of *X. oryzae* pv. *oryzae* that might be important for pathogenicity. This article is part of a Special Issue entitled: Trends in microbial proteomics.

Biological significance
We use 2D-DIGE to compare the proteomes of rice-pathogenic xanthomonads. We identify seven proteins that are co-regulated with the type III secretion system. We find post-translational glutamate methylation of a bacterial adenylate cyclase. The newly identified HrpX-regulated proteins might be important for pathogenicity.

1. Introduction

The Gram-negative gamma-proteobacterium *Xanthomonas* infects a wide spectrum of host plants, among them many economically important plants, such as rice, wheat, banana, beans, tomato, *Citrus* and cotton. *Xanthomonas oryzae* pv. *oryzae* is the causal agent of bacterial leaf blight of rice, a disease that is causing dramatic damages on rice cultivation with great economic impact around the world [1]. *X. oryzae* pv. *oryzae* is a vascular pathogen, colonizing rice leaves after

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entrance by natural pores or wounds. A better understanding of the virulence mechanisms during interaction with plants is mandatory for long-term food security.

Like many other pathogenic bacteria, *X. oryzae* pv. *oryzae* evolved a type III secretion system (T3SS) the presence of which is absolutely required for pathogenicity. This widely conserved secretion system serves as a molecular syringe and translocates so-called type III effectors (T3Es) directly into the cytoplasm of host's cells at the benefit of the pathogen [2,3]. Many T3Es interfere with cellular functions of the host's cells in order to overcome the resistance process and to accomplish the pathogen's infection cycle [4]. A 31.3-kb *hrp* (hypersensitive response and pathogenicity) gene cluster has been identified in the chromosome of *X. oryzae* pv. *oryzae* strain KACC10331, and the structure was similar to those of other characterized *Xanthomonas hrp* gene clusters [5]. Most of the 26 genes encode components of the type III secretion machinery, while others might play a role in regulation of secretion [6–13].

Expression of *Xanthomonas hrp* clusters is activated *in planta* [14] and in minimal media, such as XOM2 [15,16]. Two regulatory genes, *hrpG* and *hrpX*, which are located elsewhere in the chromosome, have been identified to control *hrp* cluster expression. HrpG is a member of the OmpR family of two-component response regulators and controls, in most cases via the AraC-type regulator HrpX, the *hrpG* regulon, including T3E genes [14,17–20]. In addition to the T3SS and T3Es, *hrpG* and *hrpX* regulate other pathogenicity genes, such as type II-secreted enzymes [15,21]. To activate gene expression, HrpX binds to a conserved *cis*-regulatory element in the promoter region, the plant-inducible promoter (PIP) box, with the consensus sequence TTCGC–N_{15}–TTCGC [17,22]. With the increasing availability of near-complete genome sequences, HrpX-regulated genes could be predicted and confirmed [17,23,24]. However, due to the degenerate nature of the PIP box, the number of false-positives and false-negatives is undesirably high, and indirect targets are not accessible by this approach.

Recently, the role of *in planta*-induced genes or *hrpG/hrpX* regulons has been studied by omics approaches, thus allowing to identify new candidate pathogenicity factors. Transcriptomic analysis by an oligonucleotide microarray revealed that in the *hrp*-inducing medium XOM2, 247 genes belonging to 17 functional categories were differentially expressed in comparison to *X. oryzae* pv. *oryzae* cells grown in rich medium [25]. Particularly, *hrpX* and other *hrp* genes were overexpressed in the minimal medium. Following the infection process of rice by an African *X. oryzae* pv. *oryzae* strain, using an SSH (subtractive hybridization) DNA microarray, over a period of time of six days demonstrated significant changes in the expression of at least 710 genes, indicating significant changes in metabolism and uncovering new candidate virulence factors [26]. The *xps* (xanthomonas protein secretion) T2SS is another essential pathogenicity factor of *X. oryzae* pv. *oryzae* [27], and the implication of *hrpG* and *hrpX* in the regulation of *xps* genes has recently been confirmed [21], highlighting the importance of these two regulatory genes in the virulence of *Xanthomonas* species.

Proteomics has been employed for several plant pathogens, including *Xanthomonas* (for review, see [28]). Some of them focused on secreted proteins and identified putative plant cell wall-degrading enzymes, among other proteins belonging to several functional categories [29,30]. Two others studies identified differentially expressed proteins of *X. axonopodis* pv. *citri* [31] or of *X axonopodis* pv. *passiflorae* [32] upon addition of leaf extract to the culture medium. In 2003, Mehta and Resato developed a method to study gene expression of *X. axonopodis* pv. *citri* cells *in vivo* within their host plant [33]. A similar approach was used to identify differentially expressed proteins of *X. campestris* pv. *campestris* upon extraction from *Brassica oleracea*. First, *X. campestris* pv. *campestris* cells grown in vitro were compared with those extracted from the plant, using two-dimensional gel electrophoresis (2DE), thus identifying in vivo expressed proteins during infection of the host plant [34]. Later, the proteome *X. campestris* pv. *campestris* cells extracted from susceptible plants was compared with that of the bacteria extracted from a resistant plant [35]. These experiments revealed major global changes in the protein pattern during interaction with resistant or susceptible host plants. In a more specific approach, *hrpX* (called *hrpB* in *Burkholderia*)-regulated proteins were discovered in the proteome of *Burkholderia glumae* cells overexpressing the regulatory protein [36]. Using 2DE and ESI-MS/MS, 34 secreted and 12 intracellular proteins were found to have a dramatic variation in abundance between overexpressing cells and the control strain. Using knockout mutants in the type III and type II secretion system, it could be demonstrated that *hrpB* does regulate not only type III- but also type II-secreted proteins.

Here, we employed 2D-DIGE to discover new proteins of *X. oryzae* pv. *oryzae* that are under control of HrpX. Thanks to the availability of a draft genome sequence of strain BAI3 in our laboratory (unpublished results), LC–MS/MS analysis allowed to identify four induced and three repressed proteins, among them two different peptidyl-prolyl *cis-trans* isomerases and an adenylate kinase. Interestingly, the adenylate kinase was found to be post-translationally modified and to exist in two different states depending on the activity of HrpX. This is the first description of a post-translational modification of a *Xanthomonas* protein that could play a role in pathogenicity.

2. Materials and methods

2.1. Bacterial strains, growth conditions and plasmids

X. oryzae pv. *oryzae* cells of the African strain BAI3 [37] were cultivated at 28 °C on PSA (1% peptone, 1% sucrose, 0.1% glutamic acid, 1.5% agar) plates or in liquid nutrient broth (NB — 1.5% peptone, 0.3% yeast extract, 0.6% NaCl, 0.1% D(+)glucose). DNA transformation of *X. oryzae* pv. *oryzae* with derivatives of pBBR1MCS-5 [38] was done by electroporation, as described before [39]. The *hrpX* plasmid was constructed from the *hrpX* gene of *X. campestris* pv. *vesicatoria* and led to the expression of a protein variant with an N-terminal hexahistidine tag and a C-terminal Strep tag which could complement an *hrpX* mutant *X. campestris* pv. *vesicatoria* [40].

2.2. RT-PCR experiments

Semi-quantitative RT-PCR was performed on *hrpC1* and *hrpD1* genes; the expression of which is known to be regulated by *hrpX*. Upon harvest of bacterial cells from 10-ml overnight

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