The inter-kingdom solo OryR regulator of *Xanthomonas oryzae* is important for motility

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SUMMARY

The LuxR-type transcriptional regulator OryR of the rice pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a member of a subgroup of regulators found in plant-associated bacteria that are known to respond to plant signals. OryR has been shown previously to positively regulate the neighbouring *pip* gene and to be important for rice virulence. The role of this inter-kingdom signalling regulator was investigated through a genome-wide transcriptome analysis. OryR was found to positively regulate 220 genes, whereas 110 were down-regulated. A significant over-representation of movement-related genes among the positively regulated ones was found, including 30 flagellar genes, accounting for 14% of the up-regulated genes above the two-fold cut-off value. In *Xoo*, both swimming and swarming respond to rice macerate and OryR plays a role in the induction of both of these types of motility under these conditions. In this study, we have also shown that the flagellar regulator *flhF* contains a *lux* box-like element in its promoter region, similar to the *oryR*-regulated neighbouring *pip* gene; via the use of a transcriptional fusion reporter, it was shown that *flhF* is regulated by OryR. Finally, the role of OryR in motility was also demonstrated by the significant reduction in flagellin content in the *oryR Xoo* mutant with respect to the wild-type, as observed by *in planta* proteomics studies.

INTRODUCTION

The population-wide regulation of gene expression in a cell density-dependent manner is known as quorum sensing (QS) (Fuqua *et al.*, 2001). The first QS system in Gram-negative bacteria was observed in *Vibrio fischeri* (Nealson *et al.*, 1970), which possesses an autoinducer-producing LuxI protein and a response regulator LuxR. As in many Gram-negative bacteria, *V. fischeri* utilizes *N*-acyl homoserine lactones (AHLs) as its signal or autoinducer molecule. This system has become a paradigm for QS in bacteria (Waters and Bassler, 2005). At high cell density and, consequently, quorum concentrations of AHLs, the LuxR–AHL complex binds at specific sequences in gene promoters, called *lux*-boxes, leading to the regulation of transcription of target genes.

An interesting twist to the archetypal AHL-QS mechanism is the widespread presence of solo LuxR-type proteins; these are transcriptional regulators having the typical modular structure of QS LuxR proteins which are not paired with a cognate autoinducer synthase (Patankar and Gonzalez, 2009b; Subramoni and Venturi, 2009). The occurrence of LuxR solos is widespread among Gram-negative bacteria. An analysis of 265 proteobacterial genomes has shown that 68 have a LuxI/LuxR system; of these, 45 contained more putative AHL-binding LuxRs than LuxIs and another set of 45 genomes contained LuxR solos (Case *et al.*, 2008). Some examples of these solos include QscR of *Pseudomonas aeruginosa*, which responds to endogenously produced AHLs (Chugani *et al.*, 2001; Fuqua, 2006; Ledgham *et al.*, 2003), and SdiA of *Escherichia coli* and *Salmonella enterica*, which responds to exogenous AHLs (Ahmer, 2004; Ahmer *et al.*, 1998; Michael *et al.*, 2001). Interestingly, there is a subgroup of LuxR solos, which is very closely related to the AHL-binding QS LuxR proteins, found in plant-associated bacteria that respond to an unknown plant signal. These solos share an interesting feature: they have one or two substitutions in the first two amino acids, which are known to be important for the binding of the autoinducer and are highly conserved in the autoinducer-binding domain of LuxR-type proteins (W₅₇ and Y₆₁ with respect to TraR) (Nasser and Reverchon, 2007). The first of these proteins to be identified was XccR in *Xanthomonas campestris* (*Xcc*). This solo regulates the expression of the adjacent gene encoding for the virulence factor PIP (a proline-imino-peptidase) in a plant signal molecule-dependent manner (Zhang *et al.*, 2007). A similar scenario to XccR of *Xcc* is found in the closely related rice pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). *Xoo* is a Gram-negative bacterium that causes leaf blight disease on the rice plant (*Oryza sativa*); it is a vascular pathogen that enters the plant leaves through the hydathodes and, once inside, divides and produces large amounts of exopolysaccharides (EPSs) that clog the xylem system. *Xoo* has a LuxR solo called OryR, which is important for rice virulence. *Xoo* does not have a LuxI synthase in its genome and does not produce AHLs. Importantly, biochemical studies have shown that OryR is not solubilized in the presence of a wide variety of AHLs, but is solubilized in the presence of media supplemented with macerated rice leaves (Feruaga *et al.*, 2007); this indicates that OryR most probably binds a plant-produced compound. Similar to XccR, OryR also regulates *pip* gene transcription in response to an unidentified rice signal molecule, and the response increases further if macerated rice,

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Fig. 1 Determination of *pip* promoter activation by OryR. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was grown in rich PYS medium (open boxes and bars) or PYS medium + rice macerate (filled boxes and bars). β -Glucuronidase levels (bars) and optical density at 600 nm (OD_{600}) (boxes) were measured at different time points. Maximum *pip* induction was found at $OD_{600} = 2.0$ M.U., Miller units; WT, wild-type.

previously infected with *Xoo*, is used as inducer (Ferluga and Venturi, 2009). Recently, a new member of this family of transcriptional regulators, the solo XagR, has been shown to be important for virulence and to regulate adhesion in *Xanthomonas axonopodis* (Chatnaparat *et al.*, 2012). Closely related solo proteins to OryR, XccR and XagR are present in nonpathogenic bacteria; for example, NesR of *Sinorhizobium meliloti*, which is a Gram-negative bacterium that can be found free living in soil or as a symbiont of alfalfa. *Sinorhizobium meliloti* has multiple QS systems synthesizing and responding to a wide range of AHLs (Gonzalez and Marketon, 2003; Marketon and Gonzalez, 2002); NesR is not involved in the production or response to AHLs, but is involved in stress adaptation and competition for nodulation (Patankar and Gonzalez, 2009a). More recently, the OryR-related solo PsoR of *Pseudomonas fluorescens* has been reported to be involved in the regulation of inhibitors of plant pathogens, including a chitinase, iron metabolism and biosynthetic genes of the antifungal compound 2,4-diacetylphloroglucinol (DAPG). Interestingly, the PsoR response seems to be plant specific (Subramoni *et al.*, 2011).

In this study, we have further analysed the role of OryR in the important rice pathogen *Xoo*. The regulon of OryR was determined through the use of genome-wide transcriptome analysis. This regulator was found to be important in the regulation of motility-related phenotypes. OryR regulates the *flhF* flagellar regulator promoter and was found to be important for swimming and swarming in the presence of rice macerate, highlighting its importance in inter-kingdom signalling.

RESULTS

Determination of the OryR regulon by genome-scale transcriptome analysis

It has been established previously that the solo OryR responds to an unknown low-molecular-weight rice molecule, triggering the transcription of the adjacent *pip* gene (Ferluga and Venturi, 2009). It

was of interest to determine whether and which other gene(s) was transcriptionally regulated by the OryR–plant molecule complex. In order to establish the growth stage to be used for the purification of RNA for transcriptome analysis, we employed a *Xoo* (pPIP122) reporter strain in which the OryR target *pip* promoter was fused to a promoterless β -glucuronidase gene in plasmid pPIP122 (Ferluga and Venturi, 2009). Maximum levels of β -glucuronidase activity were observed when *Xoo* was grown in medium with the addition of rice macerate (PYS + Rice) to an optical density at 600 nm (OD_{600}) of 2.0 (Fig. 1). The *Xoo oryR* mutant harbouring the *pip* transcriptional fusion (pPIP122), however, remained inactive at all times (data not shown). Therefore, we conclude that, as the OryR–plant compound complex maximally activated the *pip* target gene when the bacteria were grown to an OD_{600} of 2.0, in the presence of macerated rice, these growth conditions should be used to determine the other OryR gene targets.

RNA was purified from two biological replicates of wild-type *Xoo* XKK.12 (pPIP122) and *oryR* mutant *Xoo* XKK.12ORYR (pPIP122) cultures grown in PYS rice medium at OD_{600} of 2.0; prior to RNA purification, the β -glucuronidase activity was established in order to confirm the activity of OryR (data not shown). Transcriptome analysis revealed that OryR is a global regulator, influencing the transcription levels of 330 genes by two-fold or more, representing 8% of the protein coding genes in *Xoo*. OryR positively regulated 220 of these genes, whereas 110 were down regulated (Tables S1 and S2, see Supporting Information). A set of 22 genes distributed among the positively and negatively regulated genes was chosen for validation with semi-quantitative reverse transcription-polymerase chain reaction (SQ RT-PCR), conferring comparable results with the transcriptomic data (Table 1). In order to check for possible polar effects in the *oryR* insertion mutant, SQ RT-PCR analysis was performed on the *oryR* flanking genes (Xoo1267, Xoo1269 and Xoo1270); no polar effects were detected (Table 1).

Positive regulation by OryR

It was established that 220 genes (the cut-off of a two-fold increase in expression was chosen) were positively regulated by

Table 1 Validation by semi-quantitative reverse transcription-polymerase chain reaction (SQ RT-PCR) of various differentially expressed genes.

Gene ID	Gene name	Microarray fold difference	SQ RT-PCR fold difference*	P value†
<i>Positively regulated genes</i>				
Xoo3196	PilY1	32.56	13.60 ± 3.73	<0.0001
Xoo0329	Polyvinylalcohol dehydrogenase	25.83	40.70 ± 8.60	0.0093
Xoo0031	Hypothetical protein	10.18	2.26 ± 0.03	0.0063
Xoo2580	Flagellar hook-associated protein flgL	5.70	2.46 ± 0.01	<0.0001
Xoo2622	CheY	4.57	6.15 ± 1.99	0.0040
Xoo2581	FliC	4.02	3.96 ± 1.03	<0.0001
Xoo2619	FlhF	2.67	3.67 ± 0.06	<0.0001
Xoo0094	Hypothetical protein	2.36	3.15 ± 0.26	0.0002
Xoo0088	HrpB3	2.20	2.87 ± 0.39	0.0166
Xoo0752	Putative transposase	2.14	1.82 ± 0.18	0.0010
Xoo2696	Hypothetical protein	2.09	1.68 ± 0.49	0.0035
Xoo3164	Putative transposase	1.79	2.54 ± 0.13	<0.0001
Xoo2761	Internal membrane transposase	1.55	2.04 ± 0.16	<0.0001
<i>Negatively regulated genes</i>				
Xoo1701	Hypothetical protein	-34.75	-40.50 ± 2.54	0.0003
Xoo1580	RhsD protein	-5.02	-6.84 ± 0.02	0.0004
Xoo0281	Cellulase	-4.29	-1.78 ± 0.30	0.0203
Xoo3033	Hypothetical protein	-3.09	-3.80 ± 0.2	<0.0001
Xoo0869	Hypothetical protein	-3.26	-2.36 ± 0.14	<0.0001
Xoo2423	Carbonic anhydrase	-2.61	-3.85 ± 0.62	0.0002
Xoo2925	Hypothetical protein	-2.11	-2.43 ± 0.41	0.0005
Xoo2664	Gaa	-2.05	-1.81 ± 0.05	<0.0001
Xoo3227	NADH dehydrogenase subunit I	-2.00	-1.85 ± 0.13	0.0515
<i>Genes flanking oryR (Xoo1268)</i>				
Xoo1267	Amino acid transporter	1.15	-1.23 ± 0.55	0.0017
Xoo1269	Proline-imino-peptidase	1.55	2.61 ± 0.15	0.0002
Xoo1270	Amino acid transporter	-1.07	1.33 ± 0.73	0.0036

*Mean from three independent RNA isolations ± standard deviation (SD).

†Two-tailed Student *t*-test.

OryR (Fig. 2a, Table S1). The largest percentage of positively regulated genes correspond to hypothetical proteins (28%); 31% of *Xoo* KACC10331 open reading frames (ORFs) are hypothetical proteins, which is a consistent number for most of the sequenced prokaryotic genomes (Karaoz *et al.*, 2004). A closer analysis of these hypothetical proteins with the STRING tool (Snel *et al.*, 2000) showed that most are conserved among bacteria; some even have homologues in groups as distant as eukaryotes and Archaea. Interestingly, three proteins, HrpE (XOO0076), XOO0105 and XOO2584, are shared only with its close relative *Xcc* and another four, XOO4700, XOO4725, XOO4755 and XOO4791, are unique to *Xoo* [see Table S3 (Supporting Information) for the predicted functions of the hypothetical proteins]. Cell process and metabolism genes are the second most represented set of genes (25%).

A significant finding was the over-representation of movement-related genes among the positively regulated list; there were 30 flagellar genes, accounting for 14% of the up-regulated genes above the two-fold cut-off value ($P < 0.01$). Considering that, in *Xoo*, most flagellar genes are found in a single cluster of about 40 ORFs (i.e. from XOO2565 to XOO2621; Lee *et al.* 2003), this represents a major genetic locus of OryR regulation (Fig. 3). The genes positively regulated by the OryR–

plant molecule include flagellum organization and biogenesis genes (GO:0043064) (*flgE*, *flgF*, *flgG*, *flgH*, *flgI*, *flgJ*, *flgK*, *fliC*), motor activity genes (GO:0003774) (*flgB*, *flgC*, *flgE*, *flgF*, *flgG*, *flgH*, *flgI*, *flgK*, *fliF*, *fliG*, *fliJ*, *fliK*, *fliM*, *fliN*) and important regulators, such as *flhF* and *fliA*. The two flagellar genes *motC* (XOO2830) and *motB* (XOO2831), which are found in a chemotaxis cluster downstream of the main flagellar cluster, were also regulated by OryR.

In addition, there were nine chemotaxis-related genes, six of which were found in close genomic proximity to the main flagellar cluster, two in the above-mentioned chemotaxis cluster (XOO2836 and XOO2848) and one in a different chemotaxis cluster further upstream (XOO1468), which were also regulated by OryR. Furthermore, there were five pili-related genes, including *pilY1* (XOO3196) and *pilE1* (XOO3195), which were the two most strongly induced genes. Overall, 20% of all the genes positively regulated by over two-fold by OryR were motility-related genes, providing a clear indication of the role of this transcriptional regulator.

Finally, there were 17 up-regulated transposases (8%); this could be a result of the large amount of insertion sequence (IS) elements found so far in sequenced *X. oryzae* genomes, which account for up to 10% of total genes, a feature that has been

online tool (Dehal *et al.*, 2009).

One hundred and ten genes

metabolism genes. Again, hypothetical proteins are highly repre-

X004019) and a peptidase (X001259).

OryR is important for swimming and swarming in a rice-dependent manner

Following the result from the transcriptome analysis (see above), it was of interest to determine the possible role of OryR in the motility of *Xoo*; the ability of *Xoo* wild-type and *oryR* mutant to swim and swarm was therefore assayed. Swim plates indicated that both the wild-type and the *oryR* knockout mutant were motile in swimming medium and displayed the typical circular dissemination pattern from the point of inoculation. Both strains showed a slow spread through the plates, reaching a diameter of about 20 mm in 4 days. Interestingly, when macerated rice was added to the swimming medium, there was a dramatic increase in swimming by the wild-type strain, reaching a diameter of about 35 mm in 4 days, i.e. almost a 40% increase compared with medium lacking rice macerate. The importance of OryR in swimming was observed when macerated rice was included in the medium, as *oryR* mutants swam significantly less ($P = 0.0096$) (Table 2).

Swarming motility assays showed a similar trend. In this case, however, neither the wild-type nor the mutant showed swarming motility on swarming medium after 14 days (Table 2). Swarming was, however, observed when the medium was supplemented with rice macerate, being significantly reduced ($P = 0.0047$) in the *oryR* mutant (Table 2). This clearly indicated that, under the conditions tested, swarming was dependent on the presence of compound(s) present in the rice macerate and OryR played a role in this response. We attempted to complement the *oryR* mutant by introducing the *oryR* gene in both the high-copy plasmid pBBRO-ryR and the low-copy plasmid pUFR047; we then performed the swimming and swarming experiments under the same conditions. The presence of the *oryR* gene *in trans* in the plasmid did not restore the swimming and swarming phenotype to wild-type levels in either case; indeed, it decreased further the ability of the mutant to swim and swarm (data not shown). The same effect was observed when plasmid-borne copies of *oryR* were introduced in the wild-type strain. The expression of OryR in both low- and high-copy plasmids displayed very similar results; decreases of 32% and 53% in swimming motility were observed in normal medium and swimming medium complemented with rice macer-

ate, respectively. Swarming motility decreased to the same level as the complemented *oryR* mutant in medium supplemented with rice macerate (data not shown). This indicates that over-expression of *oryR* in a multicopy plasmid has adverse effects on the regulation of the gene(s) necessary for swimming and swarming. This multicopy effect has also been reported in a recent study of XagR, an OryR homologue, in *Xanthomonas axonopodis* pv. *glycines* (Chatnaparat *et al.*, 2012).

In summary, these experiments indicate that both swimming and swarming respond to rice macerate in *Xoo* under the conditions tested and that OryR plays a role in inducing both of these types of motility in the presence of rice macerate. Decreases in motility may be caused by reduced EPS production and, as xanthomonads are known to produce large amounts of EPS, the levels were measured in both the wild-type and OryR mutant. No difference in EPS production was found between the two strains (data not shown); this is in accordance with microarray data, which did not show a significant fold difference in the expression of the EPS *gum* genes.

OryR activates the *flhF* flagellar regulator

A closer look at the flagellar genetic region of *Xoo* revealed a lux-box-like element in the gene promoter of the FlhF flagellar regulator (XO02619); Although this lux-box-like element is only partially homologous to that present in the OryR-regulated *pip* promoter (Fig. 4), a relatively high degree of plasticity has been observed in QS lux-boxes (Schuster *et al.*, 2004). The FlhF regulator is known to be important for motility, as knockout mutants show a considerably decreased motility in *Xoo* (Shen *et al.*, 2001). This regulator has been reported to possess an RpoN/FleQ-dependent promoter and to be under the regulation of these master regulators in *Xcc* (Hu *et al.*, 2005; Yang *et al.*, 2009). The presence of a lux-box-like element in the promoter region of *Xoo* and the regulation of this gene as detected in the microarray experiment led us to believe that OryR plays a role in *flhF* regulation. In order to test whether this flagellar regulator is regulated by OryR, its promoter region was cloned in the pSS122 reporter plasmid (Feruaga and Venturi, 2009), giving rise to pSS123 plasmid, which was introduced into XKK12 wild-type and the derivative *oryR* mutant. β -Glucuronidase activity was monitored at different optical densities in both PYS and PYS with rice leaf macerate added. This experiment indicated that this regulator has very low activity in rich PYS medium for both strains but, when the medium was supplemented with rice macerate, the activity increased to a maximum of three-fold (Fig. 4b) at an OD₆₀₀ of 2.0. More importantly, this increase in gene promoter activity was dependent on OryR, as it remained near baseline levels in the OryR mutant. Promoter activity was restored to statistically similar levels by complementation with a plasmid-borne *oryR* gene.

Table 2 Swimming and swarming motilities of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) wild-type (WT) and *oryR* mutant strains in regular motility medium (SM) vs. medium supplemented with rice macerate. The diameters of the haloes were measured and the values are given.

Strain	Medium	<i>Xoo</i> WT	<i>Xoo oryR</i> –
Swimming (mm)	SM	22.0 $\pm$ 0.7 ^a	17.8 $\pm$ 2.0 ^b
	SM + Rice	34.6 $\pm$ 5.6 ^b	27.0 $\pm$ 6.3 ^d
Swarming (mm)	SM	–	–
	SM + Rice	65.7 $\pm$ 4.0 ^c	31.3 $\pm$ 9.6 ^e

Mean from three independent experiments $\pm$ standard deviation (SD). Values with different letters are significantly different ($P < 0.05$; analysis of variance).

Fig. 4 (a) Promoter region of the *flhF* gene with a putative *lux*-box-like element (bold) centred around position -72 from the transcriptional start site. (b) β -Glucuronidase assay of the *Xanthomonas oryzae* *flhF* promoter in *Xoo* wild-type, *oryR* insertion mutant and *oryR* complemented mutant grown in PYS and PYS + rice. M.U., Miller units; WT, wild-type; *Xoo*, *Xanthomonas oryzae* pv. *oryzae*. Means with different letters are significantly different ($P < 0.05$; *t*-test). (c) Alignment between *Xoo pip* and *flhF* boxes.

Analysis of *in vivo* *Xoo*-expressed proteins

Xoo initially enters and spreads along the rice leaf through the vascular system. In order to gain insight into the *in planta*-expressed movement-related secreted proteins, infected vascular xylem sap was collected as described in the Experimental procedures section. These samples were then submitted to proteomics analysis, revealing a set of *Xoo* proteins that were expressed during the infection. This methodology therefore allows the identification of proteins expressed by *Xoo in planta* and also permits the quantification of their relative abundance; this approach was used to further verify/validate the microarray data obtained in this study, with particular attention given to secreted and flagellar-related systems. *Xoo* wild-type- and *oryR* mutant-infected xylem saps were collected from rice 14 days post-infection; the bacteria were removed by centrifugation and the remaining proteins were subjected to proteomics analysis, after the amount of protein had been standardized through a Bradford assay (data not shown). In

the wild-type-infected vascular sap samples, the identified proteins included various well-studied virulence factors, such as 1,4- β -cellobiosidase (YP_202674), cysteine protease (YP_200126) (Furutani *et al.*, 2004), chorismate mutase (YP_199373) (Degraasi *et al.*, 2010), a lipase/esterase (YP_202009) (Rajeshwari *et al.*, 2005), a cellulase (YP_198920) (Furutani *et al.*, 2004; Sun *et al.*, 2005), among others (Table S5, see Supporting Information). Many intracellular proteins were also detected, probably as a result of cell lysis. Interestingly, flagellin (YP_201220) was the second most abundant protein in the list, with a protein abundance factor (PAF) (Powell *et al.*, 2004) of 2970.3. PAF provides semi-quantification of protein abundance, and utilizes the total number of nonredundant spectra that correlate significantly with each cognate protein (McAfee *et al.*, 2006). Flagellin, however, was found at position 50 with a four-fold decrease in relative abundance in xylem sap from *oryR* mutant-infected plants (Table S6, see Supporting Information). This decrease is in accordance with the 4.02-fold difference observed in the transcriptomics analysis and the 3.96-fold differ-

ence observed with SQ RT-PCR. In addition, a decrease was also observed in the flagellar hook protein FlgE (Xoo2572). As most of the other proteins in the list exhibited similar abundances between wild-type and the OryR mutant, this is most probably the result of the regulation of flagellin by OryR, rather than differential growth rates between the wild-type and *oryR* mutant strains.

DISCUSSION

OryR of *Xoo* is a LuxR-type transcriptional regulator, a member of a novel subgroup of plant-associated bacterial solo proteins that respond to plant signal molecule(s) (Ferluga *et al.*, 2007; Ferluga and Venturi, 2009; Subramoni *et al.*, 2011; Zhang *et al.*, 2007). OryR, like its homologous QS LuxR proteins, is composed of two domains: an autoinducer-binding domain in the N-terminus and a DNA-binding helix–turn–helix domain at the C-terminus. Interestingly, in the autoinducer-binding domain of OryR, some key amino acids present in AHL-binding QS LuxR proteins are not conserved; it is very likely that, for this reason, OryR cannot bind to AHLs, but rather interacts and responds to a different molecule(s).

In this study, we have investigated the role of OryR in the regulation of target genes in response to plant signal molecule(s). Transcriptomic studies have revealed that the largest number of genes regulated by OryR correspond to hypothetical proteins; this is not surprising given the large number of proteins of unknown function found in bacterial genomes, which can range from 30% to 40% of the total genome (Karaoz *et al.*, 2004). Of these, 11 are unique to *Xoo* and could be interesting for future research. Importantly, however, the regulation of a very large set of motility-related positively regulated genes caught our attention and was investigated further. These included flagellar, chemotactic and pili genes (Fig. 3, Table S1). Flagellar regulation is a multistep complex process; in xanthomonads, this regulation is a three-step variation of the well-studied single polar flagellum. A four-step regulation system is found, however, in vibrios and pseudomonads (Dasgupta *et al.*, 2003). The three-step regulation system in xanthomonads has been studied mainly in *Xcc* (Hu *et al.*, 2005; Lee *et al.*, 2003; Yang *et al.*, 2009); class I genes include the master regulators RpoN2 (σ^{54}) and FleQ, and the repressor FlgM, which coordinate the expression of class II genes, including those of the flagellar type III secretion system (F-T3SS) and basal body hook structure, and regulators such as FlhA (σ^{70}), FleN and FlhF. These, in turn, regulate the class III genes, which include those encoding the motor and chemotactic proteins, flagellin and the flagellar cap protein. Interestingly, the OryR positively regulated genes identified here do not include the class I master regulators RpoN2 and FleQ, which are known to be expressed constitutively (Claret and Hughes, 2000). Nonetheless, OryR positively regulated genes include those encoding the F-T3SS basal body hook structure, motor proteins, flagellar cap and flagellin (*flhC*) itself, representing a very significant percent-

age of the OryR regulon (Fig. 2a). On analysis of the promoter regions of various flagellar genes, a lux-box-like element was found centred around position –72 upstream of the translational start site of the *flhF* flagellar regulator. It was found that this promoter had very little activity in rich medium, but showed a sharp increase in activity in medium supplemented with rice plant macerate. Importantly, this increase was shown to be OryR dependent (Table 2). In *Xoo*, FlhF was identified in a yeast two-hybrid screen of a genomic library and was found to be important, but not absolutely required, for motility (Shen *et al.*, 2001); our data are in accordance with these results (Table 2).

It is believed that OryR acts predominantly in the presence of a rice signal molecule(s); thus, its role needs to be studied in medium supplemented with rice macerate or directly *in planta*. Furthermore, a recent study in *Xcc* has shown the presence of a repressor, termed XerR, that inhibits *XccR* in culture medium, but this repression is relieved *in planta* (Wang *et al.*, 2011). This was similar to our observations when analysing the swimming and swarming phenotypes in solid growth medium (Table 2), i.e. *Xoo* requires rice macerate in the medium in order to swim and swarm. The regulation of flagella by OryR in the presence of rice macerate most probably plays an important role in the observation that swimming and swarming are affected in the *oryR* mutant. As flagella are known to be important for virulence (Josenhans and Suerbaum, 2002), this may also explain our previous observation of a decreased virulence in the *oryR* mutant (Ferluga *et al.*, 2007). In this study, we have shown that the flagellar regulator *flhF* contains a lux-box-like element, just like the *pip* gene, and is regulated by OryR. FlhF has been shown to be required for the correct placement of polar flagella in *Pseudomonas putida* (Pandza *et al.*, 2000); in addition, FlhF, together with FlhG, regulates the number of flagella in *Vibrio alginolyticus* (Kusumoto *et al.*, 2006). In *P. aeruginosa*, FlhF is required for swimming and swarming and, together with FleN, orchestrates the correct placement and number of flagella (Dasgupta *et al.*, 2000; Murray and Kazmierczak, 2006). The exact role of FlhF has not been elucidated in xanthomonads, although the correct placement of the flagellum could explain the deficiency in motility observed in the OryR mutant.

It has been observed previously that bacteria possess 'intensity switches' that allow them to sense the environment in order to ensure the correct activation of flagellar and T3SS-encoding genes (Blocker *et al.*, 2003). This could indicate that *Xoo* needs to 'wait' for the correct conditions, i.e. close proximity to the hydathode, for example, in order to initiate the energy expensive process of flagellation, through the regulation of FlhF and other flagellar genes by OryR *in planta*. This is also demonstrated by motility assays, which show limited swimming and no swarming in normal motility plates but, when rice macerate is added to the medium, both phenotypes are greatly enhanced (Table 2), indicating the need for this external stimulus to start the process. This highlights

that these forms of motility are triggered by compound(s) present in the rice plant; OryR plays a role in swarming and swimming under these conditions as motility is reduced, but not eliminated. It should therefore be noted that other mechanisms also exist in *Xoo* which respond to plant signals and trigger these forms of movement. The introduction of *oryR* in a plasmid in the *Xoo oryR* mutant did not result in the complementation of this phenotype; the reason for this is currently unknown, but this phenomenon has also been observed in the complementation of phenotypes regulated by XagR, an OryR homologue, in *X. axonopodis* (Chatnaparat *et al.*, 2012). However, it has been reported previously that the introduction of multiple copies of the *oryR* homologue *psrO* of *P. fluorescens* results in unexpected behaviour/phenotypes (Subramoni *et al.*, 2011). Finally, the role of OryR in motility has also been demonstrated by the significant reduction in flagellin content in the OryR mutant observed in the *in planta* proteomics studies. This could mean that the OryR mutant is 'deaf' to the signal and thus cannot sense the correct conditions for motility to take place. As *Xoo* is a vascular pathogen, the *in planta* proteomics approach employed here, using infected xylem sap samples, proved to be an effective technique to study the proteins expressed *in vivo*.

As *Xoo* is a vascular pathogen of rice, for a successful infection, *Xoo* must: (i) move towards the host; (ii) establish contact with the host; (iii) penetrate the plant; and (iv) proliferate once inside (Gnanamanickam *et al.*, 1999). Most studies have focused on steps (iii) and (iv). *Xoo* is known to invade rice plants by entering through hydathodes, as shown by electron microscope studies (Horino, 1984; Mew *et al.*, 1984), or wounds; the latter is common during periods of high precipitation when plants are frequently damaged. Once inside, *Xoo* activates its artillery of virulence factors (for a review, see Niño-Liu *et al.*, 2006). However, the ecology of *Xoo* is poorly understood; very little is known about the functions that contribute to epiphytic growth, preference for portal of entry and modes of dissemination (Ryan *et al.*, 2011). The OryR homologue *Psor* of *P. fluorescens* is also involved in inter-kingdom signalling, probably allowing this beneficial bacterium to 'sense' its proximity to plants in order to produce antimicrobial compounds in locations in which they are useful (Subramoni *et al.*, 2011). *Xoo* could use a similar mechanism during the initial steps of infection. Once *Xoo* reaches the plant, it initially grows epiphytically (Ryan *et al.*, 2011); then, it must move in order to find a point of entry; *Xoo* is known to have a chemotactic response to water drops exuded by the rice plant (Feng and Guo, 1975). Water drops are found in wounds or hydathodes; the latter are involved in guttation, the process by which plants exude water in order to move nutrients up from the roots. It is reasonable to assume that these drops, coming from the vascular system of the plant, contain a large number of plant secondary metabolites, including the molecule to which OryR binds/responds. By sensing this molecule, *Xoo* can activate hypermotility via OryR regulation, in order to

successfully find a point of entry and colonize the plant, instead of wasting resources when not needed. In the later stages of infection, once inside the plant, *Xoo* duplicates and secretes large quantities of EPS, important in the initiation of biofilms (Ryan *et al.*, 2011), probably making flagellar motility less relevant. Importantly, and in line with our previous findings, our results show that OryR is activated and acts only in the presence of rice macerate, further highlighting its role in inter-kingdom signalling. Future studies should focus on the identification of the plant signal molecule(s) which interacts with OryR, as this will help in our understanding of the role of OryR and related proteins in plant-associated bacteria.

EXPERIMENTAL PROCEDURES

Bacterial strains, plasmids and media

All strains, plasmids and primers used in this study are listed in Table S7 (see Supporting Information). *Xanthomonas oryzae* pv. *oryzae* strains XKK.12 and XKK.12ORYR (and derivatives) were grown at 30 °C in either PYS (per litre: 8 g peptone, 2 g yeast extract, 2 g K₂HPO₄, 0.5 g KH₂PO₄, 0.25 g MgSO₄·7H₂O, 10% glucose w/v) or PS (Tsuchiya *et al.*, 1982) medium. *Escherichia coli* strains DH5α and S17-1λ-pir were grown in LB (Miller, 1972) medium at 37 °C. Medium containing macerated rice material was prepared as follows: 20–25 g of leaves were macerated in the presence of liquid nitrogen, added to 400 mL of PYS medium and autoclaved. This medium was filtered and used for the experiments. The plasmid pGEM-T Easy Vector (Promega, Madison, WI, USA) was used for cloning. Antibiotics, when required, were used at the following concentrations: ampicillin, 75 µg/mL (*X. oryzae*), 100 µg/mL (*E. coli*); kanamycin, 50 µg/mL (*X. oryzae*), 100 µg/mL (*E. coli*); gentamicin, 10 µg/mL (*E. coli*), 15 µg/mL (*X. oryzae*); rifampicin, 100 µg/mL (*X. oryzae*).

Recombinant DNA techniques

All DNA manipulations and the transformation of *E. coli* were performed as described previously (Sambrook *et al.*, 1989). Plasmids were purified using EuroClone columns (EuroClone S.p.A., Sizio, Italy) and total DNA from *X. oryzae* was isolated by sarkosyl-pronase lysis, as described previously (Better *et al.*, 1983). A transcriptional fusion construct for the *flhF* promoter in pSS122 (Furluga and Venturi, 2009) was made by amplifying a 473-bp fragment containing the *flhF* promoter region—from *X. oryzae* XKK.12 genomic DNA—using the primers flhFKpnIFw and flhFXbaIRv. This fragment was cloned into pGEM, yielding pGEMflhFprom; this plasmid was verified by sequencing (Macrogen, Seoul, South Korea) and the fragment was subsequently cloned into pSS122 (*KpnI/XbaI*), yielding pSS123. Complementation of *Xoo oryR* mutants was performed using the pBBRORYR plasmid, which was constructed as follows: a 793-bp fragment containing the *oryR* gene of *Xoo* XKK.12 was amplified using primers OryRxpBBR_fw and OryHindIII_rv, and cloned into pGEM-T Easy, yielding pGEMOryR. The fragment was subsequently cloned into the high-copy plasmids pBBR1MCS-5 and pBBR1MCS-3 (*XhoI-HindIII*) (Kovach *et al.*, 1995), yielding pBBRORYR and pBBRORYR2. The same procedure was

used for complementation in the low-copy plasmid pUFR047, employing a 1111-bp fragment containing the *oryR* gene with its native promoter region (*Bam*HI-*Hind*III) using the primers OryRF and OryRHindIII_Rv (Table S7). All plasmids were verified by sequencing (Macrogen)

β -Glucuronidase-based reporter gene fusion assay

β -Glucuronidase activities were determined as described previously (Ferluga and Venturi, 2009) in *X. oryzae* XKK.12 wild-type and *oryR* mutant, carrying the pPIP122 or pSS123 plasmids; activity was measured at various OD₆₀₀ values in PYS medium and PYS supplemented with rice (cultures were started at OD₆₀₀ = 0.1). All experiments were performed in triplicate, and the mean values are given.

Total RNA isolation

RNA isolations were carried out from cultures of *X. oryzae* strains carrying pSS122 grown in PYS medium and PYS medium supplemented with rice. The cultures were incubated (30 °C, 180 rpm) until they reached an OD₆₀₀ value of 2.0. RNA isolation was carried out from 2×10^9 cells using the Ribopure™ bacteria RNA isolation kit (Ambion Inc., Austin, TX, USA), following the manufacturer's instructions. Isolated RNA was treated with DNase at 37 °C for 1 h and purified following the directions given (Ambion). The purity of RNA was assessed by PCR on total RNA (250 ng) with GoTaq polymerase (Promega) using primers specific for *X. oryzae* KACC. RNA quality and concentration were assessed by Nanodrop (Thermo Scientific, Wilmington, DE, USA).

Microarray experiment and analysis

A custom microarray for *X. oryzae* (Roche-NimbleGen OID22515) was designed and manufactured by Roche-NimbleGen Inc. (Madison, WI, USA) in a 385-K (385 000 probe platform) chip based on the genome sequence of *X. oryzae* KACC10331 (NC_006834). The array detects a total of 3931 ORFs; 10 probes were designed per gene (when possible) and placed on the chip nine times. This adds up to a total of 39 296 specific probes. The empty spaces were filled with random (negative) probes. Microarray analyses were performed under the following conditions: (i) wild-type *Xoo* XKK.12 (pPIP122) grown in PYS medium supplemented with rice macerate; (ii) *Xoo* XKK.12 OryR⁻ (pPIP122) mutant grown in PYS medium supplemented with macerated rice. Two biological replicates of RNA samples were collected for each of the two conditions, as described above. Labeling, hybridization and scanning were performed by Roche NimbleGen, Inc. Briefly, slides were hybridized with cyanin-3 (Cy-3)-labelled samples and scanned with a NimbleGen MS200 microarray scanner. Data analysis was performed using raw fluorescence intensity values with microarray suite version 5.0. Gene functions were annotated from the National Center for Biotechnology Information (NCBI) database. Data analysis was carried out using ArrayStar software (DNASTAR, Inc., Madison, WI, USA).

SQ RT-PCR and analysis

Reverse transcription was performed in a 20- μ L reaction mixture containing 2.5 μ g of total RNA, 200 ng of random primers/ μ g of RNA (Promega) and

30 U of AMV reverse transcriptase, following the manufacturer's instructions. Conditions used for RT were 65 °C for 3 min, 25 °C for 10 min, 42 °C for 90 min and 70 °C for 10 min. The Xoo16s_Fw and Xoo16s_Rv primers were used to measure the transcription of 16S rRNA. Second-strand synthesis was performed using GoTaq Flexi polymerase (Promega) with 1 μ L of undiluted (any test gene) or 1:100 diluted (16S rRNA) cDNA reaction as the template. The number of PCR cycles for each gene was standardized so that the product amplification was in the linear range; 10 μ L of the PCR was analysed by agarose gel electrophoresis. The intensities of the bands were measured and normalized to that of 16S rRNA using ImageJ software (Abramoff *et al.*, 2004) to obtain the fold difference (the primers used are listed in Table S7). Each gene was validated twice by RT-PCR analysis of RNA samples from three independent isolations. In order to check for possible polar effects in the *oryR* insertion mutant, SQ RT-PCR analysis was performed on the *oryR* flanking genes (Xoo1267, Xoo1269 and Xoo1270); RNA transcripts were detected for all genes.

Motility assays

Fresh colonies from peptone sucrose agar (PSA) plates were stabbed into swim plates (per litre: peptone, 0.3 g; yeast extract, 0.3 g; agar, 3 g) (Shen *et al.*, 2001) and swim plates supplemented with rice macerate (5%). For swarming, 2 μ L of liquid culture (standardized to OD₆₀₀ = 2.0) were pipetted onto 4% agar M8 swarming plates with glucose as carbon source (Kohler *et al.*, 2000) and the same M8 glucose swarm plates supplemented with rice macerate (5%). The plates were incubated at 30 °C and the diameter of swimming was measured after 4 days. All experiments were performed in triplicate, and the mean values and standard deviations are given.

Rice infections and preparation of *in vivo* *Xoo* protein samples for proteomics analysis

The infection of rice plants with *Xoo* XKK.12 and xylem sap collection were performed as follows: *Xoo* was grown in PYS liquid medium at 28 °C for 2 days before being used for the infection of rice plants. The bacterial concentration was adjusted to 1×10^9 colony-forming units (cfu)/mL with sterile demineralized water prior to the inoculation of rice plants. Infection was carried out on the Italian rice cultivar Baldo, which showed high sensitivity to *Xoo* XKK.12 infection (Ferluga *et al.*, 2007). Rice was grown and infections were performed as described previously (Degrassi *et al.*, 2010). Three to four leaves per plant were inoculated using the clipping method (Kauffman *et al.*, 1973) and water was used as a negative control. Inoculated plants were kept for 24 h in humid chambers (>92% relative humidity) at 30 ± 4 °C, and were then transferred to glasshouse conditions (26–28 °C, 65% relative humidity with a photoperiod of 16 h of light and 8 h of darkness) for disease development for an additional 13 days. Infected leaves were then used for the sampling of xylem sap as follows: infected plants were placed in a humidity chamber and the dried blighted part of the infected leaves was cut 2 cm from the edge of infection. Drops oozing from the xylem were constantly collected using a 200- μ L pipette during the subsequent 4 h, placed in sterile Eppendorf tubes and centrifuged at 16 000 *g* at 4 °C for 30 min to remove bacterial cells. Xylem sap samples were pooled from the collection of bacteria from 30 plants for each sample (approximately 500 μ L for each sample), the amount of protein was standardized through Bradford assay (Bradford, 1976) and the protein was used for proteomics analysis via mass spectroscopy.

Mass spectroscopy (MS) analysis of xylem sap samples

Sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of xylem samples was used to remove the sugars and other nonproteinaceous compounds found in xylem sap; the collected xylem sap was diluted with 1 × Laemmli sample buffer, boiled for 5 min and then run onto 4%–10% Nupage gel (Invitrogen, Carlsbad, CA, USA). The samples were allowed to run 1 cm into the gel and the gel was then stained with colloidal Coomassie blue (Pierce Thermo Fisher Scientific, Rockford, IL, USA). The stained area of the gel was cut into five bands and processed for in-gel digestion with trypsin using standard procedures. The resulting peptides were cleaned up using STAGE-tips. Liquid chromatography and matrix-assisted laser desorption/ionization (LC-MALDI) of the digests was performed using an Applied Biosystems (Foster City, CA, USA) 4800 mass spectrometer coupled with an Ultimate 3000 HPLC (Dionex, Idstein, Germany) via a PROBOT target spotter (Dionex). The peptides were separated using an in-house-packed column (20 cm × 75 µm) containing Jupiter proteo resin (Phenomenex, Torrance, CA, USA). The gradient ranged from 5% to 50% acetonitrile in 0.1% trifluoroacetic acid (TFA) in 90 min. The gradient was developed at 300 nL/min and subsequently mixed with a 3-mg/mL solution of α -Cyano-4-hydroxycinnamic acid (CHCA) flowing at 1 µL/min, and spots were acquired every 10 s using a PROBOT plate spotter (Dionex, Idstein, Germany). The top 10 precursor ions from each spot were subjected to MS/MS analysis with 1500 laser shots and a laser intensity of 2600.

MGF files were created using TS2Mascot.exe (MatrixScience, www.matrixscience.com) with the mass range parameter set to a minimum of 60 Da and a maximum mass equal to the mass of the precursor. Only monoisotopic peaks with a signal-to-noise ratio > 10 were included in the final MGF.

The MGF files were searched using the X!tandem search engine (version 2009.04.01.1). Searches were conducted using a precursor mass error of 100 ppm and a fragment mass error of 0.4 Da, complete carbamidomethylation of cysteines, partial oxidation of methionine and partial deamidation of asparagine and glutamine. All data files were searched against *Xoo* strains KACC10331 (NC_006834), MAFF_311018 (NC_007705) and PX099A (NC_010717). The reverse database sequences were concatenated to the forward sequences in order to estimate the false discovery rate (FDR). Only proteins with FDR < 5% were included in the analysis.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Functional classification of OryR positively regulated genes. Only genes with a fold difference of two or greater are included.

Table S2 Functional classification of OryR negatively regulated genes. Only genes with a fold difference of two or greater are included.

Table S3 Predicted functions and domains of OryR positively regulated hypothetical proteins.

Table S4 Predicted functions and domains of OryR negatively regulated hypothetical proteins.

Table S5 *In planta* proteomics analysis of *Xanthomonas oryzae* pv. *oryzae* (Xoo) wild-type strain. Relative abundance of expressed proteins. Relative abundance values are estimated using the protein abundance factor (PAF) (see text).

Table S6 *In planta* proteomics analysis of *Xanthomonas oryzae* pv. *oryzae* (Xoo) *oryR* mutant strain. Relative abundance of expressed proteins. Relative abundance values are estimated using the protein abundance factor (PAF) (see text).

Table S7 Strains, plasmids and oligonucleotides used in this study.