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A LuxR homologue of *Xanthomonas oryzae* pv. *oryzae* is required for optimal rice virulence

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SUMMARY

In Gram-negative bacteria a typical quorum sensing (QS) system usually involves the production and response to acylated homoserine lactones (AHLs). An AHL QS system is most commonly mediated by a LuxI family AHL synthase and a LuxR family AHL response regulator. This study reports for the first time the presence of a LuxR family-type regulator in *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), which has been designated as OryR. The primary structure of OryR contains the typical signature domains of AHL QS LuxR family response regulators: an AHL-binding and a HTH DNA binding motif. The *oryR* gene is conserved among 26 *Xoo* strains and is also present in the genomes of close relatives *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri*. Disrupting *oryR* in three *Xoo* strains resulted in a significant reduction of rice virulence. The wild-type *Xoo* strains do not seem to produce AHLs and analysis of the *Xoo* sequenced genomes did not reveal the presence of a LuxI-family AHL synthase. The OryR protein was shown to be induced by macerated rice and affected the production of two secreted proteins: a cell-wall-degrading cellobiosidase and a 20-kDa protein of unknown function. By expressing and purifying OryR it was then observed that it was solubilized when grown in the presence of rice extract indicating that there could be a molecule(s) in rice which binds OryR. The role of OryR as a possible *in planta* induced LuxR family regulator is discussed.

INTRODUCTION

Bacteria which belong to the genus *Xanthomonas* are Gram-negative belonging to the Gammaproteobacteria and are significant pathogens to a large number of plants worldwide (Vandamme *et al.*, 1996). One member of the genus is *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), which is the causal agent of a serious disease in rice called bacterial leaf blight (Swings *et al.*, 1990). This disease causes severe losses and is most predominantly found in tropical Asian countries. It is a vascular disease whereby *Xoo* continues to grow until the xylem vessels are clogged with bacterial cells and extracellular polysaccharides. In the last 15 years several studies have improved our understanding of the molecular determinants of rice/*Xoo* interaction with the cloning of several rice resistance (*Xa*) genes, *Xoo* avirulence (*avr*) genes and the hypersensitive response and pathogenicity (*hrp*) genes (Leach and White, 1996; Leach *et al.*, 2001; Shen and Ronald, 2002). Importantly, *Xoo* consists of a diversity of races which exhibit different virulence, thus making the breeding of durable resistant rice cultivars a major challenge. Recently, the genomes of two *Xoo* strains have been completely sequenced, annotated and published (Lee *et al.*, 2005; Ochiai *et al.*, 2005).

In most bacteria a major level of regulation involves intercellular communication via the biosynthesis and response to signal molecules (Camilli and Bassler, 2006). It is a cell-density-dependent regulation of gene expression which has been termed quorum sensing (QS) (Fuqua *et al.*, 1994). QS provides significant advantages to a community of bacteria including improving access to environmental niches, enhancing defence capabilities against other microorganisms or eukaryotic host-defence mechanisms, and facilitating the adaptation to changing environmental conditions (for reviews see Camara *et al.*, 2002; Fuqua and Greenberg, 2002; Waters and Bassler, 2005). In fact, it is most probable that in natural ecosystems bacteria are often aiming at establishing communities rather than choosing to exist as solitary cells. In Gram-negative bacteria, a typical QS system usually involves the production and response to an acylated homoserine lactone (AHL). The AHL-dependent QS system is commonly mediated by two proteins belonging to the LuxI-LuxR families (Fuqua and Greenberg, 2002). LuxI-type proteins are responsible for synthesizing AHLs from *S*-adenosyl methionine and particular fatty acyl carrier proteins. AHLs then interact directly, at quorum concentration, with the cognate LuxR-type protein and this protein-AHL complex can then bind at specific gene promoter sequences called *lux*-boxes affecting expression of QS target genes. AHL QS has been the subject of extensive investigation in recent years

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and has become a paradigm for bacterial intercellular signalling. Other QS signalling molecules have been discovered which are produced by Gram-negative bacteria, including a quinolone signal molecule produced by *Pseudomonas aeruginosa* and a molecule designated AI-2 (4,5-dihydroxy-2,3-pentanedione, DPD), which is produced by a wide range of bacteria (Camilli and Bassler, 2006; Waters and Bassler, 2005). In addition, it is also becoming evident that bacteria can produce and respond to more than one QS signalling molecule (Camilli and Bassler, 2006).

To our knowledge, in the genera *Xanthomonas* there are no reports of the presence of AHL QS systems. By contrast, QS has been reported in *Xanthomonas campestris* pv. *campestris* and *Xoo* to occur via a signalling molecule designated DSF (diffusible signal molecule) (Chatterjee and Sonti, 2002; Dow *et al.*, 2003; He *et al.*, 2006). DSF has been characterized as *cis*-11-methyl-2-dodecenoic acid synthesized by the RpfF protein (Barber *et al.*, 1997); DSF signalling is involved in the regulation of biofilm dispersal and production of virulence factors. Current studies support the role of a two-component regulatory system designated RpfC/RpfG in the perception and transduction of the DSF signal to target genes (Dow *et al.*, 2003; He *et al.*, 2006). In the present study we investigated whether *Xoo* produces and responds to AHLs and present evidence (1) that a set of *Xoo* isolates do not produce AHLs, (2) for the existence of a conserved LuxR QS regulator in *Xoo* which we designated OryR, (3) that OryR is important for rice virulence, (4) that OryR is induced by macerated rice and (5) that OryR most probably interacts with a plant signal.

RESULTS AND DISCUSSION

Rice pathogenicity tests on *X. oryzae* pv. *oryzae* isolates

The pathogenicity of the 23 *Xoo* isolates from India was tested on the susceptible rice line IR24 and three near-isogenic lines with known resistance genes IRBB5 (Xa5), IRBB7 (Xa7) and IRBB21 (Xa21). IR24 and IRBB7 were the most susceptible lines. They were resistant only to one of the 23 tested isolates, while IRBB5 and IRBB21 were resistant to seven and ten *Xoo* isolates, respectively (Table 1).

Table 1 *Xanthomonas oryzae* pv. *oryzae* strains used.

Isolate name	State in India	Site	Year of isolation	Host cultivar	Pathogenicity tests*				Reference
					IR24	IRBB5	IRBB7	IRBB21	
XAPT.43	Andhra Pradesh	Tada	1.2003	Unknown	S	S	S	I	This study
XAPC.5	Andhra Pradesh	Cudappah	1.2003	Unknown	S	S	S	R	This study
XAPC.10	Andhra Pradesh	Cudappah	1.2003	Unknown	S	I	S	R	This study
XAPC.11	Andhra Pradesh	Cudappah	1.2003	Unknown	S	S	S	R	This study
XAPC.12	Andhra Pradesh	Cudappah	1.2003	Unknown	S	I	S	I	This study
XAPC.13	Andhra Pradesh	Cudappah	1.2003	Unknown	S	S	S	R	This study
XAPC.14	Andhra Pradesh	Cudappah	1.2003	Unknown	S	R	S	R	This study
XAPC.19	Andhra Pradesh	Cudappah	1.2003	Unknown	S	S	S	I	This study
XAPC.20	Andhra Pradesh	Cudappah	1.2003	Unknown	S	R	S	R	This study
XAPC.23	Andhra Pradesh	Cudappah	1.2003	Unknown	S	S	S	R	This study
XKK.3	Kerala	Kannanur	1.2003	Jyothi	S	S	S	I	This study
XKK.4	Kerala	Kannanur	1.2003	Jyothi	S	S	S	S	This study
XKK:12	Kerala	Kannanur	1.2003	Jyothi	S	S	S	S	This study
XKK.16	Kerala	Kannanur	1.2003	Jyothi	S	S	S	R	This study
XKPt.4	Kerala	Palghat	1.2003	ADT.46	S	I	S	S	This study
XKPt.8	Kerala	Palghat	1.2003	ADT.46	S	I	S	S	This study
XKP2.2	Kerala	Parali	1.2002	Matta Tiruvani	S	R	I	R	This study
XP4.2	Kerala	Pattambi	9.1999	Jyothi	S	I	S	S	This study
XKV.5	Kerala	Valancheri	1.2003	Thiruveni	S	R	S	S	This study
XKV.9	Kerala	Valancheri	1.2003	Thiruveni	S	R	S	S	This study
XKV.15	Kerala	Valancheri	1.2003	Thiruveni	R	R	R	R	This study
XTNAi.18	Tamilnadu	Adthurai	1.2003	ADT.46	S	R	I	S	This study
XTNP.4	Tamilnadu	Podi	10.2002	ADT.46	S	S	S	S	This study
LMG5047	Unknown	Unknown	1965	Unknown	S	N/A	N/A	N/A	
BX043	Unknown	Unknown	Unknown	Unknown	S	N/A	N/A	N/A	(Goel *et al.*, 2002)
KACC10331	Korea†	Unknown	Unknown	Unknown	S	N/A	N/A	S	(Lee *et al.*, 2005)

*R; resistant, I; intermediate, S; susceptible. See text for details.

†This strain has been isolated in Korea (Lee *et al.*, 2005) and not in India (see text for details).

N/A, not available.

Even though the number of tested isolates was relatively low, our results suggested that the resistance gene Xa7 is less efficient than Xa5 and Xa21 in the concerned sites of India. However, the adult resistance earlier demonstrated in the rice–*Xoo* interaction (Qi and Mew, 1985; Sidhu and Khush, 1978) could have influenced our results and this resistance gene was possibly not expressed in 45-day-old rice plants. We believe that the group of 23 isolates tested is a good representation of *Xoo* strains, which can be used for analysis of AHL production (see below).

X. oryzae pv. *oryzae* rice pathogenic bacteria do not produce AHLs

Using bacterial biosensor AHL detector strains described in the Experimental Procedures section, all the *Xoo* isolates listed in Table 1 were used initially to test by growth in solid media in a plate streak assay for AHL production (Hwang *et al.*, 1994). The bacterial biosensor *Chromobacterium violaceum* CVO26 induces the production of violacein when certain AHL signal molecules are present, *Escherichia coli* (pSB401) and *E. coli* (pSB1075) induce bioluminescence, and *Agrobacterium tumefaciens* NT1 (pZLR4) and *Pseudomonas fluorescens* 1855 (pSF105, pSF107) induce β-galactosidase production. These four AHL biosensor strains ensure that a wide range of AHLs are detected as each displays specificity towards structurally different AHLs (Steindler and Venturi, 2007). In addition to the 24 *Xoo* strains described above we also tested the well-studied *Xoo* strain BXO43 and strain KACC10331, the genome of which has been sequenced (all listed in Table 1). All 26 *Xoo* strains gave a negative result in solid media in plate streak assays. Although this may mean that these strains do not produce AHLs at all, we could not exclude that they did so in very low amounts. In order to test this, 100 mL spent culture supernatant was extracted and analysed for AHLs by thin-layer chromatography (TLC) followed by a bioassay. The detection of AHLs in TLC plates was visualized by making use of *E. coli* JM109 (pSB401), *E. coli* (pSB1075) and *A. tumefaciens* NT1 (pZLR4) detector strains. Again all 26 *Xoo* strains were tested for presence of AHLs of spent supernatant and all gave a negative result. It was concluded that *Xoo* most probably does not produce AHL molecules. It cannot be excluded that *Xoo* could be producing AHLs at extremely low amounts that cannot be detected or the AHL biosensors used here do not respond to the AHL molecules potentially produced by *Xoo*.

The genome of *X. oryzae* pv. *oryzae* contains a potential QS orphan LuxR-family member

The genome sequences of two *Xoo* strains, designated as KACC10331 and MAFF311018, have been recently published (Lee *et al.*, 2005; Ochiai *et al.*, 2005) and a third strain, PX086, is currently being sequenced. Analysis of the genomes did not reveal any gene and/or protein which belongs to the LuxI family of AHL synthases (Fuqua and Greenberg, 2002). A second distinct, although small, family of AHL synthases has been reported and is composed of the LuxM, AinS and VanM proteins from *Vibrio harvey*, *V. fischeri* and *V. anguillarum*, respectively (Milton *et al.*, 2001). Analysing the genomes of *Xoo* again revealed that no LuxM homologue was found. These results are in accordance with our observation that no AHLs could be detected from 26 different *Xoo* isolates including strain KACC10331, the genome sequence of which was available.

Analysing the three *Xoo* genome sequences, however, revealed the presence of a shared identical LuxR family member (*Xoo* KACC10331; Q5H3E9, *Xoo* MAFF311018; Q2P6A5 and from *Xoo* PXO86; Q6R756), which we designated here as OryR, having the characteristic signatures of an AHL-dependent response regulator. OryR is 254 amino acids long and it contains an autoinducer binding domain (Pfam03472) from position 22 to 178 and HTH domain from 189 to 246 with the conserved region of LuxR family regulators (Fig. 1). Interestingly, although its function is unknown, an open reading frame (ORF) highly similar (over 90%) to OryR is

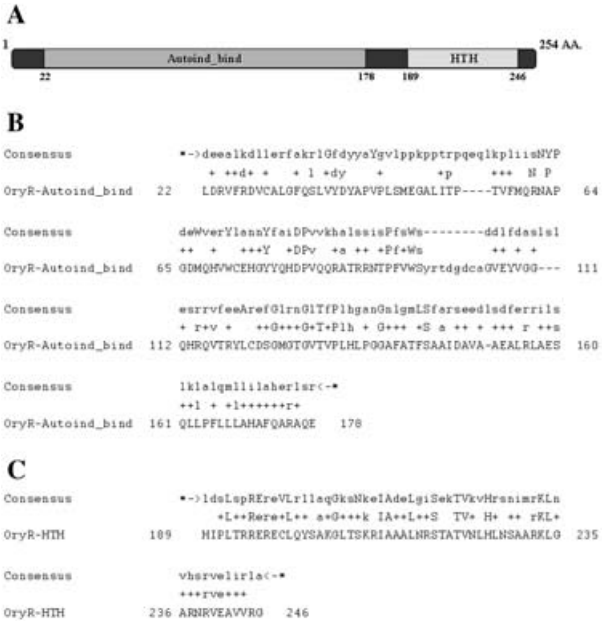

Fig. 1 The OryR protein primary structure contains domains typical of quorum sensing LuxR family regulators. (A) Schematic representation of OryR; numbers refer to amino acid residues. Positions of the AHL-binding and the HTH-DNA-binding domains are shown. (B) Alignment using the one-letter code between the consensus of the AHL binding domain (shown as consensus, Pfam03472) and (C) HTH-DNA-binding domain of LuxR family regulators and the corresponding domain in OryR. Amino acid identities of less important residues in the domains are shown as small capitals; a + sign refers to conserved amino acids with similar properties (http://www.sanger.ac.uk/Software/Pfam/).

also present in the closely related *Xanthomonas campestris* pv. *vesicatoria* (Q3BQU7), *X. axonopodis* pv. *citri* (Q8P1BO) and *X. campestris* pv. *campestris* (Q4UX59). All *oryR* genes have been annotated as single transcriptional units and not part of operons. OryR displays approximately 50% similarity to several LuxR family proteins of various *Pseudomonas syringae* plant pathogenic species (Q48E34, QOEE63, Q87WEEK7 and Q4ZNM6) and to several LuxR proteins belonging to members of the *Rhizobium* genera (Q92M41l, Q1M918 and Q2K5W3. Interestingly, all these bacteria are closely associated with plants.

OryR could therefore act as a LuxR 'orphan' (i.e. lacking a cognate *Lux*I AHL synthase) QS type protein possibly responding and regulating target genes to signals from neighbouring AHL-producing bacteria. Such examples have thus far not been commonly reported in bacteria; to our knowledge the only example is SdiA of *E. coli* and *Salmonella enterica*, which enables these bacteria, which do not synthesize AHLs, nevertheless to respond to exogenous AHLs produced by other bacterial species (Ahmer, 2004). Two other examples of orphan LuxR proteins have been reported which, in apparent contrast to OryR, respond to AHLs produced by the same cell. These proteins are QscR of *P. aeruginosa* and ExpR of *Sinorhizobium meliloti*. One proposed role of these proteins is to extend the AHL QS regulation in these bacteria to other gene targets. However, as QscR and ExpR possess a broader, more relaxed, response to a larger number of different AHLs, a second proposed role is to increase the range of AHLs to which these species respond (Hoang *et al.*, 2004; Lequette *et al.*, 2006).

oryR is conserved in *X. oryzae* pv. *oryzae*

As mentioned above, orthologues of *oryR* are present in *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri*. In order to determine if *oryR* is conserved in other *Xoo* isolates, we performed Southern blot analyses of NcoI restriction enzyme-digested chromosomal DNA of all 26 *Xoo* isolates reported in Table 1 with a DNA fragment containing the complete *oryR* gene. This probe gave a positive hybridization signal at high hybridization stringency conditions with 19 of the 26 *Xoo* isolates (Fig. 2). Of the seven which did not give a signal, we analysed by Western analysis whether they contained an OryR-like protein; five of the seven did respond to anti-OryR antibody having a protein band of the expected size (data not shown). It was concluded that *oryR* is widely and not completely conserved within the *Xoo* species.

oryR mutants of *X. oryzae* pv. *oryzae* are less virulent in rice

The *oryR* gene has been mutated in three *Xoo* strains in order to understand its role in rice pathogenicity. The three *Xoo* strains were KACC10331, the genome of which has been sequenced, *Xoo* strain BXO43, in which several molecular studies have been performed, and *Xoo* strain XKK.12, which has been reported here and is very virulent to rice (Table 1). Rice virulence analysis was performed with the three *oryR* mutants as well as their respective wild-type parent strains. The assays were performed on rice leaves at very high concentration of inoculum (10^9 CFU/mL) as well as at lower concentration (10^8 and 10^7 CFU/mL). As depicted in Fig. 3, the XKK.12 *oryR* mutants were less virulent as compared with the wild-type strain at all inoculum concentrations, indicating that OryR was necessary for optimal *Xoo* rice pathogenicity. In strains BXO43 and KACC10331, however, *oryR* mutants displayed less virulence only at high inoculum as under these conditions the wild-type strains displayed strong virulence.

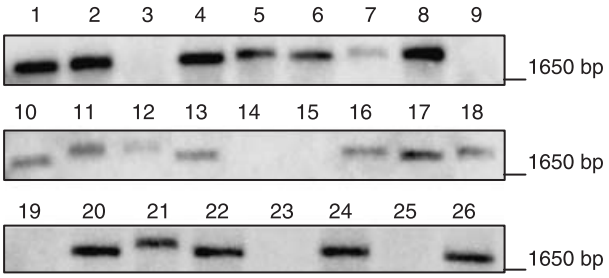

Fig. 2 High stringency Southern analysis using *oryR* DNA as a probe against *Nco*I-digested chromosomal DNA from 26 *Xoo* strains. The number correspond to the following *Xoo* strains (see Table 1 for further details): 1, XAPT.43; 2, XAPC.5; 3, XAPC.10; 4, XAPC.11; 5, XAPC.12; 6, XAPC.13; 7, XAPC.14; 8, XAPC.19; 9, XAPC.20; 10, XAPC.23; 11, XKK.3; 12, XKK.4; 13, XKK.12; 14, XKK.16; 15, XKPt.4; 16, XKPt.8; 17, XKP2.2; 18, XP4.2; 19, XKV.5; 20, XKV.9; 21, XKV.15; 22, XTNAi.18; 23, XTNP.4; 24, LMG5047; 25, BX043; 26, KACC10331.

OryR is solubilized by macerated rice

The study of LuxR family QS proteins has shown that when over-expressed they are insoluble; however, in the presence of their cognate AHL molecule, which they bind with high affinity, they become soluble. In fact, the cognate AHL is required for the proper folding of the nascent protein, for formation of homomultimers and for protection to proteases (Chai and Winans, 2004; Collins *et al.*, 2005; Schuster *et al.*, 2004; Urbanowski *et al.*, 2004; Zhu and Winans, 2001). In order to determine if the OryR protein did interact with AHLs we performed biochemical studies on the protein. By over-expressing and purifying OryR, it was established that, like other LuxR-homologue proteins, OryR was highly insoluble when overproduced in *E. coli*.

As OryR contains an Autoind_bind domain (Pfam03472, see above) it was hypothesized that if OryR was able to bind to one or more AHLs this could possibly allow OryR to solubilze. His_6-OryR was therefore expressed and purified in its native form in the presence of 20 μM of each of several non-substituted AHLs

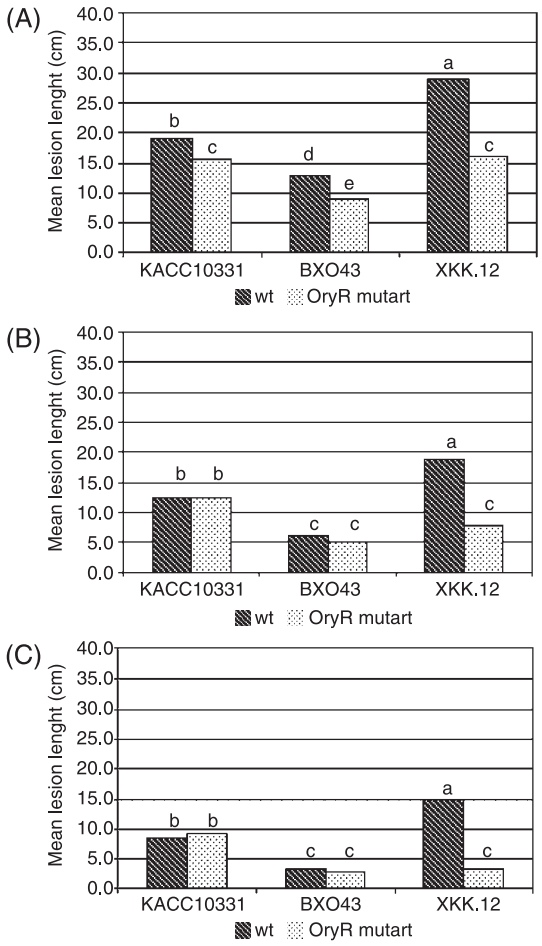

Fig. 3 *oryR* mutations affect *Xoo* virulence. Results of leaf lesion lengths caused by three *Xoo* and *oryR* mutant derivatives. Bars with different letters are significantly different using the Mann–Whitney comparison test performed on lesion length data. (A) A 10^9 CFU/mL *Xoo* inoculum was used, (B) 10^8 CFU/mL, (C) 10^7 CFU/mL. See text for details.

(i.e. C4-, C6-, C8-, C10- and C12-AHL), several 3-oxo-AHLs (i.e. 3-oxo-C6-, 3-oxo-C8-, 3-oxo-C10- and 3-oxo-C12-AHLs) and several 3-OH-AHL molecules (i.e. 3-OH-C6, 3-OH-C8, 3-OH-C10 and 3-OH-C12). No OryR protein solubilization was observed when the protein was over-expressed in the presence of these molecules (data not shown). Although the AHLs used here are the ones most commonly used in bacteria, the list is not complete, as other structural AHLs have been reported to be produced by Gram-negative bacteria.

OryR protein solubility was also studied in the presence of plant components in the growth medium. Ten grams of rice plants (leaves and stems) were frozen with liquid nitrogen and macerated; the resultant rice powder was added to LB medium. After nickel affinity chromatography, pure His-tagged OryR in native form was eluted at 135 mM of imidazole (Fig. 4B). The presence of OryR in the elution peak was confirmed by Western blot analysis using OryR antibody (Fig. 4C). As control, no OryR solubilization peak was observed in the presence of LB medium alone (Fig. 4A) or LB medium supplemented with tobacco (*Xoo* non-host plant) macerated powder (Fig. 4B); for both of these controls, no OryR was detected in a Western analysis of soluble fractions (data not shown). This result raises the hypothesis that molecule(s) specifically present in rice may possibly bind and solubilize some OryR. This could also be an indication that OryR might be active and performing gene regulation functions *in planta* (see below).

OryR is induced by macerated rice

In order to determine if OryR is expressed when *Xoo* KACC10331 was grown in laboratory media, we determined OryR levels using anti-OryR antibodies. When *Xoo* KACC10331 was grown in minimal media no OryR protein was detected with Western analysis (Fig. 5A), whereas when grown in minimal media supplemented with macerated rice, OryR levels increased significantly and could be clearly detected (Fig. 5A). These results indicate that *oryR* is most probably induced *in planta*, thus most probably affecting target gene expression when *Xoo* is in rice. OryR was not detected when *Xoo* KACC10331 was grown in minimal media in the presence of a cocktail of exogenously provided AHLs (Fig. 5A), indicating that they do not affect the expression of *oryR*. It cannot be excluded, however, that some component(s) of rice extracts from macerated rice are involved in stabilizing OryR from proteolytic degradation, thus increasing protein levels rather than affecting its transcriptional status.

In order to identify possible OryR target genes, we analysed the profile of secreted proteins of the wild-type *Xoo* KACC10331 strain vs. the profile of the *oryR* mutant KACC10331ORYR to determine if any secreted proteins were regulated by OryR. As depicted in Fig. 5B, in KACC10331ORYR two secreted proteins of approximately 60 and 20 kDa were present at lower levels when compared with the parent strain, indicating that OryR as well as rice extract was important for their production. The approximately 60-kDa protein was digested with trypsin and analysed by mass spectroscopy, resulting in the determination of the following peptides: MGNGIDAVR, SYPTYVWLDSIDAIYGGSR, QAGLQR, TEYIDVIASTLANPKYK and FLIDTGR. Performing a BLAST analysis the peptides were 100% identical to parts of secreted enzyme 1,4-beta-cellobiosidase of *Xoo* KACC10331 (NC_006834.1). This secreted enzyme designated CbsA is a cell-wall-degrading enzyme, which was recently determined to be very important for *Xoo* virulence (Jha *et al.*, 2007). The 20-kDa protein band was identified in a similar way with the following peptides, ADASSPINLLSPAARK, FVESLRFNASGVTSFR, IRVDSEEDR and VDSEEDR, and corresponded to a *Xoo* 20-kDa hypothetical protein of unknown function (YP_452683). In the putative promoter regions of these two genes, we were able to identify poor potential lux-box sequences (the 1,4-beta-cellobiosidase gene contains a putative lux box at

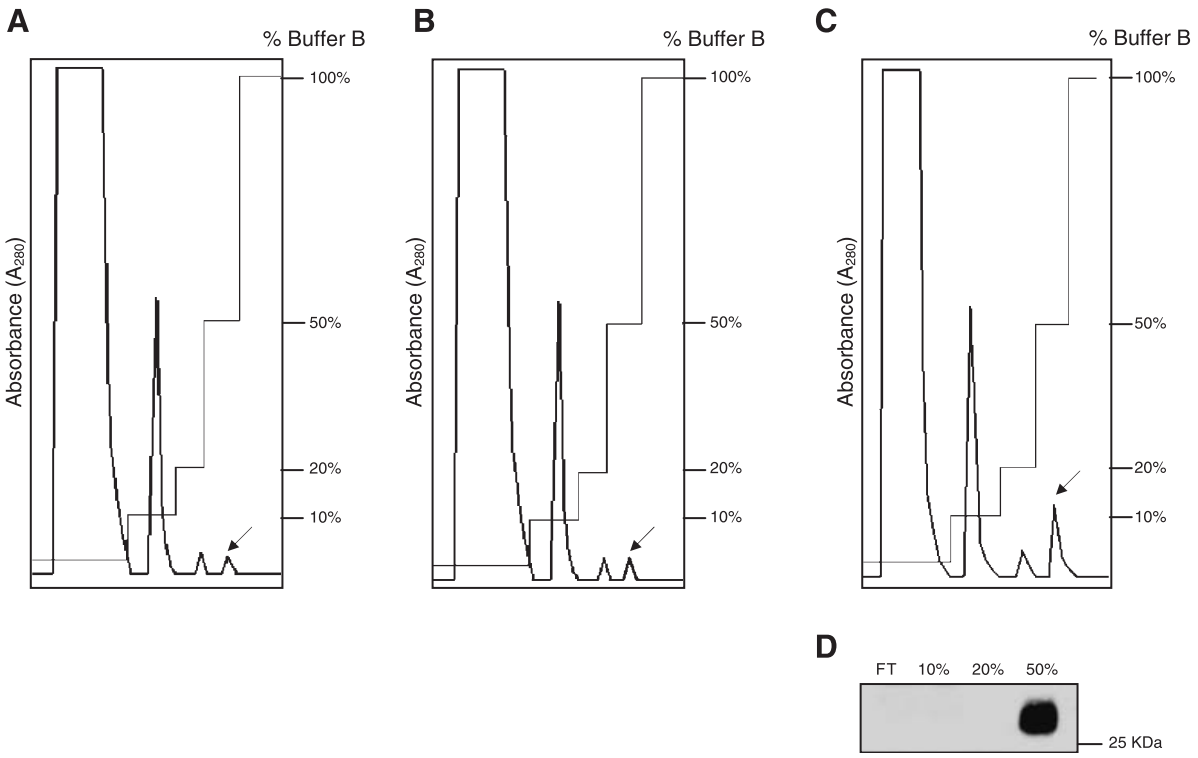

Fig. 4 Solubility studies of His6-OryR. (A) Affinity chromatography using LB as growth medium for *E. coli* harbouring pPQEORYR and pREP-4. Arrow indicates putative low amounts of OryR protein eluted at 50% of buffer B, corresponding to 135 mM of imidazole (see Experimental Procedures) or background due to *E. coli* His-rich proteins. (B) Affinity chromatography using LB supplemented with macerated tobacco as growth medium for *E. coli* harbouring pPQEORYR and pREP-4. Arrow indicates putative low amounts of OryR protein eluted at 50% of buffer B, corresponding to 135 mM of imidazole. (C) Affinity chromatography using LB supplemented with macerated rice as growth medium for *E. coli* harbouring pPQEORYR and pREP-4. Arrow indicates increase of OryR protein eluted at 50% of buffer B, corresponding to 135 mM of imidazole (see Experimental Procedures). (D) Western blot analysis of each elution peak from His6-OryR affinity chromatography (C) using anti-OryR antibody. The same result was obtained using Anti 6x-His monoclonal antibody (data not shown). FT, flow through.

position –411 to –430 relative to the translational start codon, whereas the 20-kDa protein is at position –205 to –221). Whereas, the putative promoter region of the *oryR* gene also contains a more conserved lux-box (at position –123 to –142; Fig. 6). At present, however, it is not known if any of these are functional. Lux boxes in gene promoters are binding sites for LuxR family proteins (Fuqua and Greenberg, 2002).

CONCLUDING REMARKS

This study reports for the first time the presence of a LuxR family-type regulator in *Xoo* which has been designated as OryR. The primary structure of OryR is very similar to that of domains found in AHL-responsive QS LuxR family response regulators: an AHL-binding and a HTH DNA binding motif. We have tested 26 *Xoo* strains for production of AHLs and found that none produces these signal molecules. The *oryR* gene is conserved among *Xoo* strains and is also present in close relatives *X. campestris* pv. *campestris* and *X. axonopodis* pv. *citri*. Our results show that OryR is involved in rice virulence given that three *Xoo oryR* mutant strains showed reduced pathogenicity. As OryR does not have a typical cognate AHL LuxI family synthase it could be defined as an orphan QS LuxR-type response regulator (Ahmer, 2004; Fuqua, 2006; Hoang *et al.*, 2004; Lequette *et al.*, 2006; Walters and Sperandio, 2006). Our working model is therefore centred towards the possibility that OryR responds to AHL compounds produced by other bacteria or by AHL mimic compounds derived from rice (Degrassi *et al.*, 2007). Although attempts to solubilize recombinant OryR using several different AHL compounds failed, OryR solubilization was achieved in the presence of rice extract. This suggests that OryR could respond to some molecule(s) present in rice, indicating that this regulator could be involved in inter-kingdom signalling (Shiner *et al.*, 2005). As OryR contains an AHL-binding motif it is reasonable to speculate that the molecule could be closely related to AHLs. Interestingly, OryR was present only when *Xoo* was grown in the presence of macerated rice and affected the production of two secreted proteins: a cell-wall-degrading enzyme and a protein of unknown function. AHL

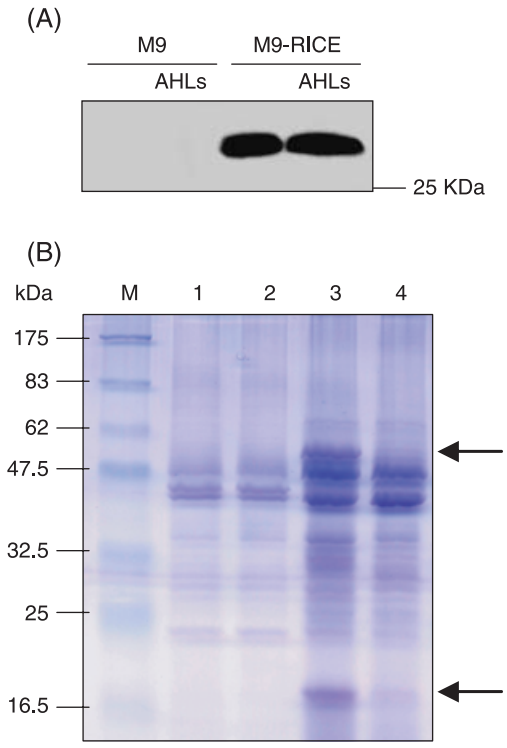

Fig. 5 (A) OryR levels in *Xoo* grown in different conditions. Similar amounts of stationary phase *Xoo* cells grown in (1) M9 medium alone or with a cocktail of AHLs (C4-, C6-, C8-, C10- and C12-AHLs, same for 3-oxo-AHLs and for 3-OH-AHLs, all added at 1 μM) and (2) M9 in the presence of macerated rice or with a cocktail of AHLs (see above). Proteins were then examined by Western analysis with anti-OryR antiserum. See text for details. (B) SDS-PAGE analysis of total secreted proteins of *Xoo* KACC10331 grown in minimal M9 medium (lane 1), *Xoo* KACC10331ORYR grown in minimal M9 medium (lane 2), *Xoo* KACC10331 grown in minimal M9 medium supplemented with macerated rice (lane 3) and *Xoo* KACC10331ORYR grown in minimal M9 medium supplemented macerated rice. The arrows indicate the two proteins only seen to be produced by the wild-type KACC10331 strain grown in the presence of macerated rice and not by the *oryR* mutant derivative.

QS is the paradigm of intercellular signalling in Gram-negative bacteria and OryR could possibly extend these systems to having roles in communication with eukaryotes. Future studies will focus on identifying *Xoo* gene targets of OryR and the molecule(s) to which it responds.

EXPERIMENTAL PROCEDURES

Bacterial strains media and plasmids

Xanthomonas oryzae pv. *oryzae* (*Xoo*) strains used are listed in Table 1. Detection of AHL signal molecules was performed using the following bacterial biosensors; *Chromobacterium violaceum* CVO26, *Escherichia coli* JM109 (pSB401), *E. coli* JM109 (pSB1075), *Agrobacterium tumefaciens* NT1 (pZLR4) and *Pseudomonas fluorescens* 1855 (pSF105, pSF107) (all reviewed by Steindler and Venturi, 2007). *Xoo* strains were routinely grown at 28 °C grown either in PYS (per litre: 8 g peptone, 2 g yeast extract, 2 g K_2HPO_4, 0.5 g KH_2PO_4, 0.25 g $MgSO_4 \cdot 7H_2O$, 10% glucose w/v) PS medium (Tsuchiya *et al.*, 1982) or in M9 minimal medium with the addition of Casamino acids (Sambrook *et al.*, 1989) and if necessary macerated cv. Baldo rice plants (rice plants were frozen at −80 °C, then macerated with a pestle and mortar). Kanamycin at 100 μg/mL was used for *Xoo* growth. *E. coli* was routinely grown in LB medium at 37 °C and antibiotics were added when necessary at the following concentrations: ampicillin, 100 μg/mL; kanamycin, 100 μg/mL; tetracycline, 15 μg/mL.

Recombinant DNA techniques

Digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 DNA ligase, end filling with Klenow fragment of DNA polymerase, Southern hybridization and transformation of *E. coli* were performed as described (Sambrook *et al.*, 1989) Analytical amounts of plasmids

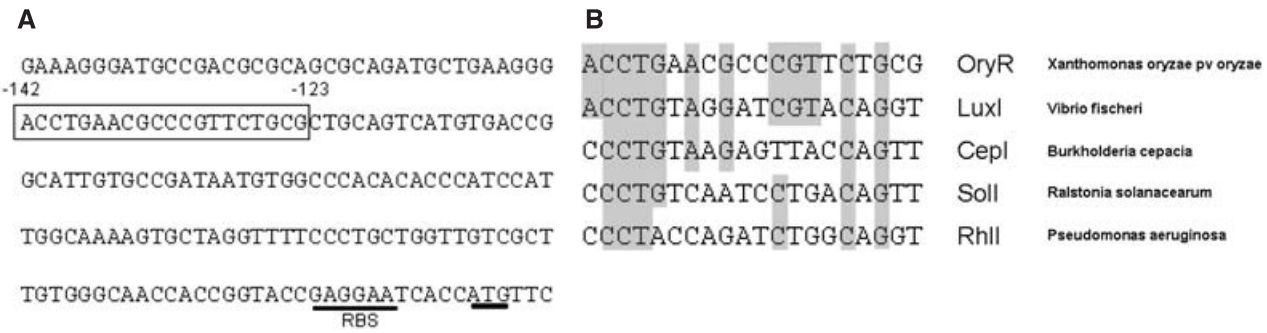

Fig. 6 Putative lux box in the *oryR* promoter region. (A) Nucleotide sequence of the promoter region of the *oryR* gene; underlined is the RBS (putative Shine Dalgarno sequence) and the ATG of the translational start codon. Boxed is the putative *lux* box and the numbers indicate the distance relative to the start codon. (B) Alignment of the *oryR* putative *lux* box with *lux* boxes in the promoter of the AHL synthases of several bacteria. Shaded are the conserved nucleotides.

were isolated as described (Birnboim, 1983), whereas preparative amounts were purified with Qiagen columns. Total DNA from *Xoo* was isolated by the sarcosyl-pronase lysis method (Better *et al.*, 1983).

Purification, detection and visualization of signal (AHL) molecules

The purification, detection and visualization of AHL signal molecules from culture supernatants were performed essentially as previously described (Steindler and Venturi, 2007, and references therein). Synthetic C4-AHL to C12-AHL were purchased from Fluka Chemie AG (Buchs, Switzerland) and C6–3oxo-AHL to C12-3-oxo-AHL and C6-3-OH-AHL to C12-3-OH-AHLs were purchased from the laboratory of Professor Paul Williams (University of Nottingham, UK).

Inactivation of *oryR* of *X. oryzae* pv. *oryzae*

The *Xoo* KACC10331 *oryR* gene was in part amplified by PCR as a 385-bp fragment using primers oryRintS, 5′-CGTCTAGAGGTGGAATATGTGG-3′, and oryRintR, 5′-ATCTCTGAGTTCAGATGCAGGT-3′, and cloned as an *Xba*I-*Xho*I fragment in pKNOCK-Km (Alexeyev, 1999) generating pKNORY. This latter plasmid was used as a suicide delivery system in order to create an *oryR* knock-out mutant in *Xoo* strains KACC10331, BXO43 and XKK.12 as described (Alexeyev, 1999) generating KACC10331ORYR, BXO43ORYR and XKK.12ORYR. The fidelity of the marker exchange events was confirmed by Southern analysis (data not shown).

OryR antibodies and protein analysis

Antibodies against OryR of *Xoo* were generated by injecting purified protein into rabbits. *Xoo* OryR was purified as His_6-OryR in pQEORYR in *E. coli* M15 (pREP-4) according to the instructions of the supplier (Qiagen, Hilden, Germany). pQEORYR was constructed as follows: *oryR* of *Xoo* KACC10331 was amplified by PCR using two oligonucleotides, oryRqes, 5′-CCCGGATCCTTCGAAATTCTA-3′, and oryRqer, 5′-ACCAAGCTTTTATGGCTCCAG-3′, and cloned as a *Bam*HI-*Hin*dIII fragment in pQE30 (Qiagen) yielding pQEORYR.

Proteins were transferred on to PVDF membrane (Immobilon-P; Millipore) using a tank system according to the manufacturer's instruction. The membrane was subjected to Western blot analysis using polyclonal antibodies against either OryR or 6x-His monoclonal antibody (BD Biosciences, San Jose, CA) and after incubation with the second HRP-labelled antibody the proteins were detected with the 3–3′-diaminobenzidine (DAB) tetrahydrochloride tablets (Sigma, St. Louis, MO). No significant cross-reaction of the polyclonal antibody against other *Xoo* or *E. coli* proteins was observed in this study.

Total secreted proteins were isolated and characterized as follows. Cells from 10-mL overnight cultures were pelleted by centrifugation for 10 min at 8000***g***. Cells remaining in the supernatant were removed by an additional centrifugation step for 3 min at 15 000***g***. Proteins in the cell-free supernatant were then precipitated with 10% (w/v) trichloroacetic acid, dried and resuspended in 40 μL of sample buffer and loaded at 20 μL in the SDS-PAGE gel.

Protein analysis of secreted and total proteins was performed by boiling the protein suspension in sample buffer for 10 min; the proteins were then separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on gels containing 12% (w/v) polyacrylamide.

Selected protein bands were identified as follows: the band was cut out from the Coomassie Brilliant blue-stained gel and placed in a siliconized microcentrifuge tubes that had been rinsed with water and ethanol. The band was digested with trypsin, and the resulting peptides were extracted with water and 60% acetonitrile/1% trifluoroacetic acid. The fragments were then analysed by mass spectroscopy (an internal sequence analysis of the protein spots was performed by using an electronspray ionization mass spectrometer LCQ DECA XP, ThermoFinnigam); proteins were identified by analysis of the peptides and by using the *Xoo* protein data banks.

OryR over-expression and purification

For OryR over-expression, a single plate colony of *E. coli* M15-pQEORYR was used to inoculate 10 mL of LB-ampicillin-kanamycin and grown overnight; 1 mL of the culture was then used to inoculate 100 mL of prewarmed medium. Expression was induced adding 1 mM isopropyl-β-D-thiogalactoside at an OD_{600} of 0.6 and carried on for 3 h at 37 °C. The culture was rapidly chilled on ice and the cells were harvested by centrifugation and frozen at –80 °C.

His_6-OryR protein was extracted under denaturing and native conditions according to the supplier's instructions (Quiagen). The purification step was performed using a 5-mL HiTrap affinity column (Amersham Pharmacia). Native His_6-OryR was eluted using a imidazole step gradient [10 mM (Buffer A) and 250 mM (Buffer B)]. Protein concentration was determined by using a Bradford assay (Bio-Rad).

Bacterial leaf blight virulence assays on rice plants

Xanthomonas oryzae pv. *oryzae* isolates were grown on Sucrose Peptone Agar medium (Tsuchiya *et al.*, 1982) at 28 °C and single colonies were transferred to liquid Sucrose Peptone medium. Two-day-old cultures were used for inoculum production. The bacterial concentration was determined using a spectrophotometer (Multiscan Ex) and adjusted to 10^9, 10^8 or 10^7 CFU/mL with demineralized water. Pathogenicity tests on the *Xoo* strains in Table 1

were carried out on rice cultivar IR24 and three NILs (Near Isogenic Lines): IRBB5, IRBB7 and IRBB21 (from the International Rice Research Institute). Germinated rice seeds were grown in trays in a potting compost (Klassmann substrate 4, Geeste, Germany) under greenhouse conditions (30 ± 4 °C) with a 16 : 8-h light–dark photoperiod. Plants were weekly fertilized with 5 g $(NH_4)_2SO_4$ and 10 g $FeSO_4 \cdot 7H_2O/m^2$. Forty-five-day-old plants were used for infection tests.

Rice plants were inoculated by the clipping method (Kauffman *et al.*, 1973). Three to four leaves were used per plant and one leaf per rice line was clipped using H_2O for control. Five to six plants were inoculated per isolate and were kept for 18 h in humid chambers (≥ 92% relative humidity) at 30 ± 4 °C, and were thereafter brought back to greenhouse conditions for disease development. Fourteen days after inoculation, symptoms were evaluated by measuring the lesion length of the leaf covered by bacterial leaf blight lesion. Plants were divided into three classes: resistant, with lesion length of 0–3 cm; intermediate, 3–9 cm; and susceptible, > 9 cm.

Rice cultivar IR24 was used to assess the virulence of *Xoo* strains KACC10331, BXO43 and XKK.12 and their respective *oryR* mutants. Twenty to 25 leaves were infected by each of the six strains. Experiments were performed in triplicate. Lesion length data did not fulfil the requirements for ANOVA and were statistically analysed in the program SPSS using the non-parametric tests: Kruskal–Wallis multiple comparison completed by the Mann–Whitney test.

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