Studies on the Inter-Cellular Communication Mechanisms in the Rice Bacterial Pathogens *Xanthomonas oryzae* pv. *oryzae* and *Pseudomonas fuscovaginae*

Thesis

Studies on the inter-cellular communication mechanisms in the rice bacterial pathogens *Xanthomonas oryzae* pv. *oryzae* and *Pseudomonas fuscovaginae*

Sara Ferluga

This thesis is submitted for the degree of Doctor of Philosophy in the faculty of Life Sciences of the Open University, UK

International Centre for Genetic Engineering and Biotechnology (ICGEB)
Trieste - Italy

Director of Studies: Vittorio Venturi, Ph.D.
Second Supervisor: John Maxwell Dow, Ph.D.

September, 2008

Submission date: 4 Aug. 2008
Date of award: 20 Oct. 2008

To Daniele

ACKNOWLEDGEMENTS

I am extremely grateful to Dr. Vittorio Venturi for allowing me to work under his enthusiastic guidance and for sharing his expertise in microbiology and in molecular biology. I also thank Dr. John Maxwell Dow for his supervision and for his inspiring ideas. I am grateful to the Examination committee, Dr. George P. Salmond and Dr. Richard M. Cooper, for their useful suggestions and constructive comments to improve this manuscript.

I would like to express my sincere gratitude to Prof. Francisco E. Baralle for giving me this stimulating opportunity to work in an international research institute and to all the administrative and technical ICGEB staff members for their daily assistance. Many thanks to Mrs. Nerina Brandolin for her logistic support during all the entire process.

I would like to express all my gratitude to all the Bacteriology group, in particular to Dr. Giuliano Degrassi, Giulia Devescovi and Iris Bertani, for their important suggestions and for the fruitful discussions. Special credits must be given to Dr. Joseph Bigirimana, for sharing his expertise in plant pathology and its practical support to this work.

There are many others who helped me in many ways through all these years, and even if I would love to, it is impossible for me to mention them all now.

Finally, my heartfelt thanks to my parents, and especially to my husband, to whom this thesis is dedicated, for their constant support and understanding during these challenging years.

ARC12
(September 2007)

RESEARCH SCHOOL

Affiliated Research Centre Programme

Library Authorisation

Please return this form to the Research School, The Open University, Walton Hall, Milton Keynes, MK7 6AA with the two bound copies of the thesis to be deposited with the University Library. All candidates should complete parts one and two of the form. Part three only applies to PhD candidates.

Part One: Candidate Details

Name: Sara Ferluga PI: X3268109

Degree: Ph. D.

Affiliated Research Centre: International Centre for Genetic Engineering and Biotechnology (ICGEB)

Thesis title: Studies on the inter-cellular communication mechanisms in the rice bacterial pathogens

Xanthomonas oryzae pv. *oryzae* and *Pseudomonas fuscovaginae*

Part Two: Open University Library Authorisation

I confirm that I am willing for my thesis to be made available to readers by The Open University Library, and that it may be photocopied. subject to the discretion of the Librarian.

Signed: Date: 17 November 2008

~~Part~~ Three: British Library Authorisation [PhD candidates only]

If you want a copy of your PhD thesis to be available on loan to the British Library Thesis Service as and when it is requested, you must sign a British Library Doctoral Thesis Agreement Form. Please return it to the Research School with this form. The British Library will publicise the details of your thesis and may request a copy on loan from the University Library. Information on the presentation of the thesis is given in the Agreement Form.

Please note the British Library have requested that theses should be printed on one side only to enable them to produce a clear microfilm. The Open University Library sends the soft bound copy of theses to the British Library.

The University has agreed that your participation in the British Library Thesis Service should be voluntary. Please tick either (a) or (b) to indicate your intentions.

(a) ☒ I am willing for The Open University to loan the British Library a copy of my thesis. A signed Agreement Form is attached.

(b) ☐ I do not wish The Open University to loan the British Library a copy of my thesis.

Signed: Date: 17 November 2008

An electronic version of this form can be downloaded from http://www.open.ac.uk/research/research-degrees/affiliated-research-centre-programme/affiliated-research-centres.php

ABSTRACT

The most common quorum sensing (QS) system in Gram-negative bacteria employs *N*-acyl homoserine lactones (AHLs) as signal molecule. AHLs allow bacteria to monitor their cell density being commonly used to synchronize/coordinate the expression of virulence-associated factors in a community. An AHL QS system is most commonly mediated by two proteins belonging to the LuxI-AHL synthase and to LuxR-AHL response regulator protein families. AHLs interact directly at quorum concentration with the cognate LuxR-type protein which then binds at QS target gene promoters affecting their transcription. The purpose of this thesis was to investigate the QS systems based on AHL signal molecules in two important bacterial rice pathogens: *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Pseudomonas fuscovaginae*. Studies revealed that *Xoo* does not produce AHLs and does not possess a *luxI* AHL synthase gene; it does have however an unpaired *luxR*-homolog gene closely related to QS *luxR* family genes which was designated *oryR*. OryR was demonstrated to be involved in inter-kingdom signalling by binding an unknown rice signal molecule (RSM) and affecting bacterial gene expression. The concentration of the RSM increases in rice when it is infected with *Xoo* possibly meaning that it is involved in a response to pathogen attack. RSM does not bind canonical LuxR-family proteins and is not related to AHLs. It was concluded that OryR is not involved in bacterial QS but in inter-kingdom signalling by recognizing and responding to a molecule present in rice. Studies in *P. fuscovaginae* revealed that it possesses a typical AHL QS system, designated PfvI/R, highly conserved within the species and highly similar to the LasI/R and PpuI/R QS systems present in *Pseudomonas aeruginosa* and

Pseudomonas putida respectively. The PfvI/R QS system was shown to be involved in virulence, to be important for the hypersensitivity response in non-host plants and for bacterial motility.

INDEX OF CONTENTS

INDEX OF PICTURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

APPENDIX

INDEX OF TABLES

INTRODUCTION

1. INTRODUCTION

1.1 General information on rice

The worldwide transformation of agriculture during the 1960s and 1970s, generally referred to as "Green Revolution", led to significant increases in agricultural production, improving crop genotypes, farm technology, better irrigation and chemical fertilizers. This transformation has occurred as the result of programs of agricultural research that resulted in the development of modern or high yielding crop varieties (Evenson and Gollin, 2003). Revolution in agriculture helped food production to keep pace with worldwide population growth. Despite its success at increasing aggregate food supply, the Green Revolution as a development approach has not necessarily translated into benefits for the lower strata of the rural poor in terms of greater food security or greater economic opportunity and well-being. How the Green Revolution affects rural people depends on whether they are wage earners, cultivators or consumers, whether they come from landed or landless, rich or poor, male- or female-headed households. Undernutrition and poverty are still prevalent and the distribution of food remains skewed with families in landless, small-scale farming households and general labourers as high-risk groups.

Rice is one of the most important crops in the world, providing staple food for more than half of the world population. Rice is in fact the most economically important food crop in many developing countries but has also become a major crop in many developed countries (IRRI - www.irri.org). Rice provides two thirds of the calorie

needs of more than 3 billion people in Asia and one third of the calorie needs of nearly 1.5 billion people in Africa and Latin America.

World production of rice has risen steadily from about 200 million tons of paddy rice in 1960 to about 650 million tons in 2007 (FAO – www.fao.org). Rice production represents 30% of the world cereal production today. It has doubled in the last 30 years, in part due to the introduction of new genetically improved varieties, but its present growth barely follows consumption: in 2025 there will be 4.6 billion people that depend on rice for their daily nourishment, compared with three billion today. A new leap in production is therefore expected. At the same time, small producers will have to use land which is less favourable for cultivation, such as brackish or briny soils, and the availability of water resources will become more and more problematic. Ninety percent of the world crop is grown and consumed in Asia. Rice is the only major cereal crop that is primarily consumed by humans directly as harvested, and only wheat and corn are produced in comparable quantity (FAO – www.fao.org; IRRI - www.irri.org).

1.2 Classification of rice

Rice is a kind of grass, member of the family Poaceae, subfamily Oryzoideae. Of the 21 known species of rice, only two are cultivated: the widely grown Asian rice, *Oryza sativa*, and the hardier African rice, *Oryza glaberrima* (Sweeney and McCouch, 2007). About 50,000 varieties exist within these two species, but only a few hundred of them are cultivated. Asian rice, if managed with modern techniques such as fertilizers, irrigation, and chemical pesticides, produces significantly more grain per plant than African rice, and for this reason is the preferred type in the majority of rice-

growing countries. African rice, however, is more productive than Asian rice in traditional farming systems where modern techniques are not used or poor growing conditions are present. Rice should not be confused with 'wild rice' (Figure 1-D) which is produced by the North American *Zizania aquatica*, also in the grass family but not closely related.

O. sativa is differentiated into three subspecies based on geographic conditions and amylose content: indica, javanica, and japonica (Sweeney and McCouch, 2007). Indica (Figure 1-A) refers to the tropical and subtropical varieties, grown throughout South and Southeast Asia and southern China, with long grains and high in amylose, cooking to fluffy grains to be eaten with the fingers. Javanica (Figure 1-B), designates the bulu (awned) and gundil (awnless) rice with long panicles and bold grains, growing in Indonesia, intermediate in amylose content and stickiness. Japonica (Figure 1-C) refers to the short and roundish grained varieties of the temperate zones of Japan, China, and Korea, low in amylase and cooking to sticky masses suitable for eating as clumps with chopsticks. Moreover rice is further divided into long, medium and short-grained varieties. Rice is also classified as white or brown, not on the basis of the variety, but according to how it is processed.

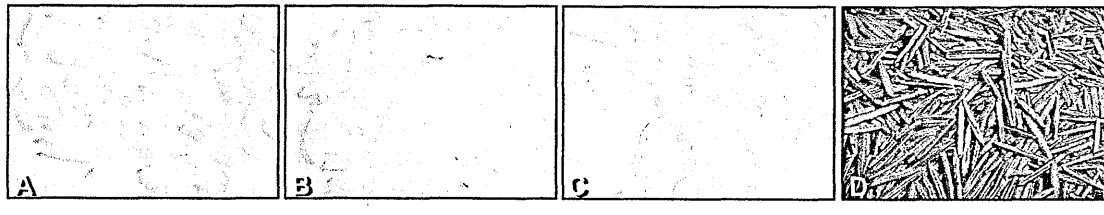


Figure 1: Rice grain varieties. Indica, with long grains (A), Javanica, with bold grains (B), Japonica, with short and roundish grains (C) and the North American wild-rice (D).

White rice undergoes a number of processes to eliminate all the external layers, while the first phase for brown rice is to remove the husk without touching the other layers. Brown rice contains the highest nutritional and mineral value. *Oryza sativa* is much more commonly used to produce rice and is cultivated from 53° N to 40° S latitude where it is adapted to a wide range of environmental conditions, from uplands to waterlogged lowlands. Rice is grown in more than 100 countries, and is particularly productive in tropical regions with abundant moisture, but it also grows successfully under widely different climates. Today, rice is grown in four different ecosystems: irrigated, rainfed lowland, upland, and flood-prone.

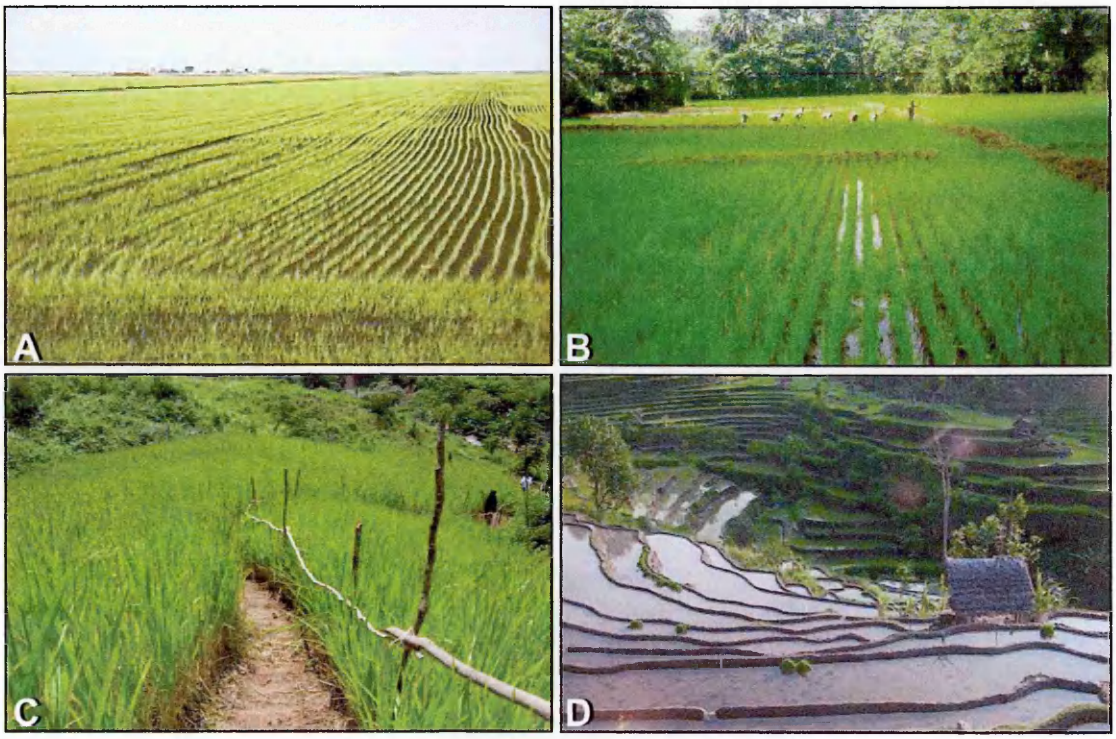


Figure 2: The four rice ecosystems characterized by different water regimes. (A) Irrigated ecosystem, in bounded field with regular water supply. (B) Rainfed lowland ecosystem, in bounded fields with irregular water supply. (C) Upland ecosystem, in unbounded well-drained fields. (D) Flood-prone ecosystem, in deeply flooded fields.

Irrigated rice is grown in levelled fields with water control, producing paddy rice that is the preferable method for commercial rice production (Figure 2-A). Rainfed lowland rice is cultivated in bounded fields non-continuously flooded with water (Figure 2-B). In the upland ecosystem rice is often grown in hilly areas with natural rainfall, upland rice produces significantly lower yields than paddy rice and requires abundant moisture at frequent intervals (Figure 2-C). Rice in flood-prone ecosystem is grown in fields subjected to temporary or long periods of submergence in floodwater. Flooding is used to control some weeds and insect pests, but may lead to waterborne disease spread, some water weeds and some water insect pests (Figure 2-D).

1.3 Structure of the rice plant

Rice is an annual plant with several jointed culms or stems, the lower part floating in water or prostrate, with roots at the nodes, and the rest erect. Cultivated species of rice are considered to be semi-aquatic. The height of the plant can range from 0.4 m to more than 5 m in some deepwater rice types. It has round, hollow, jointed stems, rather flat, sessile leaf blades and a terminal panicle. The rice plants develop new shoots (tillers) with the number of shoots depending on spacing and soil fertility. A single shoot appears first, followed by one, two and more offshoots. Each stalk has five, six or more hollow joints and a leaf is located at each joint. The leaf blades are long, pointed, flat and rather stiff. The highest joint on the plant grows a branched head called panicle (Figure 3). Each head bears from 50 to 300 flowers (spikelets) from which the grains develop. When the grain has developed, the panicle droops under the weight of the ripened kernels (Cottyn, 2003).

The growth duration of the rice plant ranges from 3 to 6 months from germination to maturity, depending on the cultivar and the environment under which it is grown. Rice passes through the following 10 stages during its growth cycle: (1) germination and emergence, (2) seedling, (3) tillering, (4) stem elongation, (5) panicle initiation, (6) panicle development, (7) flowering, (8) milk grain, (9) dough grain, and (10) mature grain stage. These stages can be summarized basically in three sequential growth periods: vegetative, reproductive and ripening.

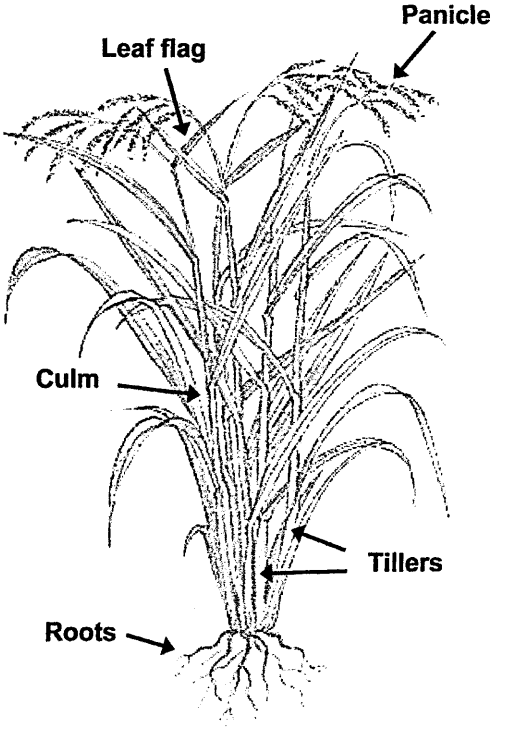


Figure 3: Structure of the rice plant (Cottyn, 2003).

The vegetative stage refers to a period from germination to the initiation of panicle primordial, and is characterized by active tillering and gradual increase in plant height. Active tillering refers to a stage when the increase in tiller number is high. A single plant can have from 16 to 25 tillers at the maximum tillering stage depending

on the cultivar, light, spacing, nutrient supply, and cultural practice. The tillers remain attached to the plant at maturity even as the individual tillers produce their own roots. The reproductive stage spans the period from panicle primordial initiation to flowering, and is characterized by culm elongation, emergence of the flag leaf (the last leaf), booting, heading (panicle emergence), and flowering. The ripening stage refers to the period from flowering to maturity, and is characterized by leaf senescence and grain growth, which may be subdivided based on grain texture and colour into milky, dough, yellow-ripe, and maturity stages (Cottyn, 2003).

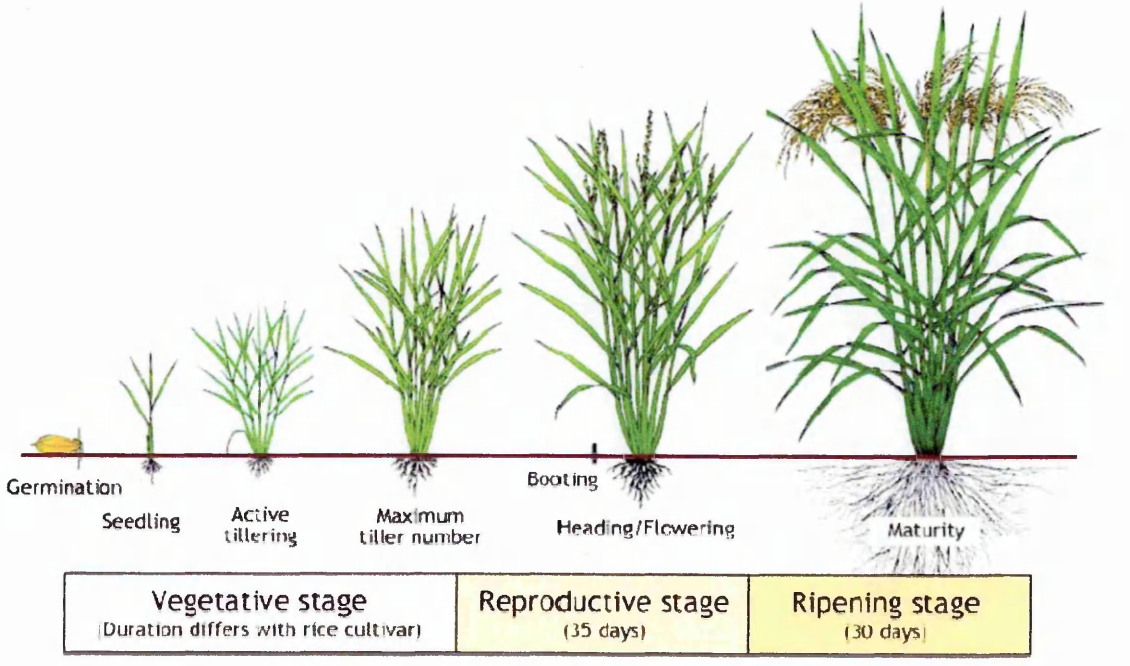


Figure 4: Growth stages of the rice plant (Cottyn, 2003).

1.4 Cultivation and harvesting

Methods of growing differ greatly in different localities, but in most Asian countries the traditional hand methods of cultivating and harvesting rice are still practiced. The

fields are prepared by plowing, fertilizing, and smoothing. The seedlings are started in seedling beds and, after 30 to 50 days, are transplanted by hand to the fields, which have been flooded by rain or river water. During the growing season, irrigation is maintained by dike-controlled canals or by hand watering. The fields are allowed to drain before cutting. Depending on the rice variety and the climate, rice grains are ready for harvest in three to six months. Rice when it is still covered by the brown hull is known as paddy; rice fields are also called paddy fields or rice paddies. A paddy is a complete seed of rice and one grain of paddy contains one rice kernel. Each paddy has many layers, the outermost layer is the husk. The husk consists of 2 interlocked half shells, each protects one half of the paddy. The next layers are bran layers. Each layer is a very thin film of bran. The fibrous bran of brown rice is rich in oil; protein; the B vitamins: thiamin, riboflavin, and niacin; and the minerals: iron, phosphorus, and potassium.

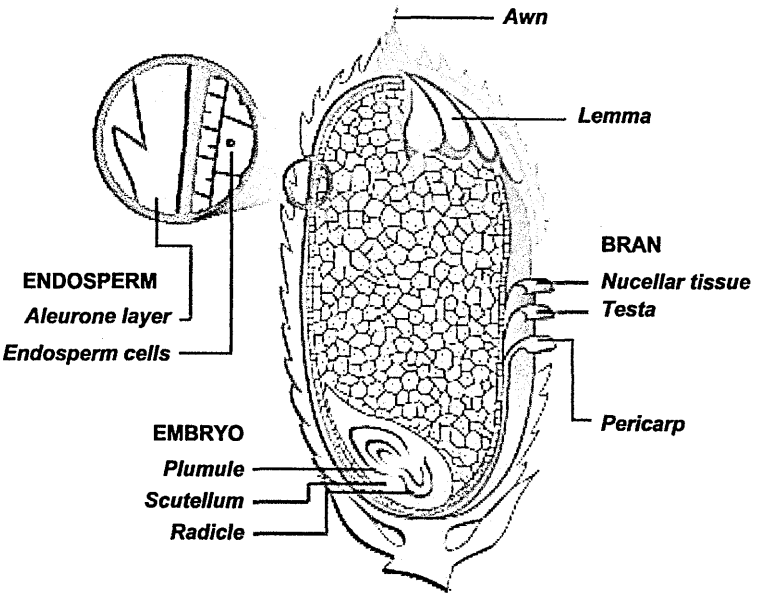


Figure 5: Structure of the rice seed (http://www.bernas.com.my/process.htm).

To make white rice, the bran is removed. White rice is less nutritious than brown rice and, when feasible, is enriched with the addition of vitamins and minerals to increase its nutritive value. At the base of each grain is an embryo, which will grow into a new plant if planted (Figure 5). The inner part of the grain is the rice kernel, which is composed of mainly starch. Rice starch is composed of mainly 2 types of starches, amylose and amylopectin. The exact mixture of these determines the cooking texture of the rice.

1.5 Diseases affecting rice

Rice is susceptible to a range of diseases and pests, which annually destroy about 55% of rice crops. The most common fungal diseases are sheath blight and rice blast, and the stalk borer is a common insect pest. Weeds compete with rice for nutrients and water and are a serious problem, especially in upland rice farming. Weeds are controlled by integrating cultural practices and herbicides. Field preparation includes complete weed removal through disking. Immediately after planting, an herbicide is applied that inhibits weed seed germination or kills growing weeds. Rodents and birds also feed on rice grains before they are harvested. Rice diseases are influenced by factors such as fertilization rate, soil type, environmental conditions and variety susceptibility. Depending upon the severity, diseases can cause substantial losses in yields as well as decreased grain quality. Growers control certain diseases by using seed treatment, resistant varieties and fungicides along with cultural and management practices.

The rice seed provides a habitat for a rich diversity of microorganisms consisting of bacteria, fungi, microscopic algae, as well as members of the microfauna such as plant

nematodes. Seed is therefore a potential vehicle for transmitting plant pathogens, but, on the other hand, beneficial microorganisms can be applied onto seeds to deliver the organisms using the seed as vehicle. The term "seedborne pathogens" includes all plant pathogenic bacteria, fungi, nematodes and viruses that can be carried out in, on or with seeds (Agarwal and Sinclair, 1997).

Considering the upper part of the plant, bacterial blight, blast, sheath blight, and sheath brown rot, are among the economically most important rice diseases worldwide.

1.5.1 **Bacterial Blight** (bacterium - *Xanthomonas oryzae* pv. *oryzae*)

The disease is one of the most important diseases of rice. Symptoms usually develop in the field at the tillering stage and the disease incidence increases with plant growth, peaking at the flowering stage. Lesions on the leaf blade are initially water-soaked and typically associated with the leaf tips and edges. The lesions gradually expand and turn yellowish and eventually greyish-white. High rainfall with strong winds are thought to provide conditions for the bacteria to multiply and enter the leaf through injured tissue (Figure 6-A).

1.5.2 **Blast** (fungus - *Magnaporthe grisea*)

This disease is one of the most devastating diseases occurring to rice, is particularly destructive in the temperate irrigated lowland and tropical upland environments. The fungus produces spots or lesions on leaves, nodes, panicles, and collar of the flag leaves. The centre of the spot is usually grey and the margin brown or reddish-brown. Both the shape and colour of the spots may vary and resemble those of the

brown leaf spot disease. Spores of the fungus are produced in great abundance on blast lesions and can become airborne, disseminating the fungus a considerable distance. Control measures include early planting, avoiding excessive or high levels of nitrogen, proper flood management, resistant varieties, and fungicides (Figure 6-B).

1.5.3 **Sheath Blight** (fungus - *Rhizoctonia solani*)

Initial symptoms usually develop as lesions on sheaths of lower leaves near the water line when plants are in the late tillering or early internode elongation stage of growth. The disease causes lesion on the leaf sheaths and the leaf blades. These lesions usually develop just below the leaf collar as oval-to-elliptical, green-grey, water-soaked spots. With age, the lesions expand and the centre of the lesions typically turns greyish-white with a brown margin. Disease development progresses very rapidly in the early heading and grain filling growth stages during periods of frequent rainfall and overcast skies (Figure 6-C).

1.5.4 **Sheath Brown Rot** (bacterium - *Pseudomonas fuscovaginae*)

Symptoms include brown necrotic lesions ranging from small specks to large brown blotches on the flag leaf sheath or extensive necrosis of the sheath, poor panicle emergence, grain discoloration and sterility of spikelets (Figure 6-D).

More than 100 pathogens among bacteria, fungi, nematodes and viruses, have been reported to attack rice, however not all of them are economically significant. The most important ones are presented in Table 1.

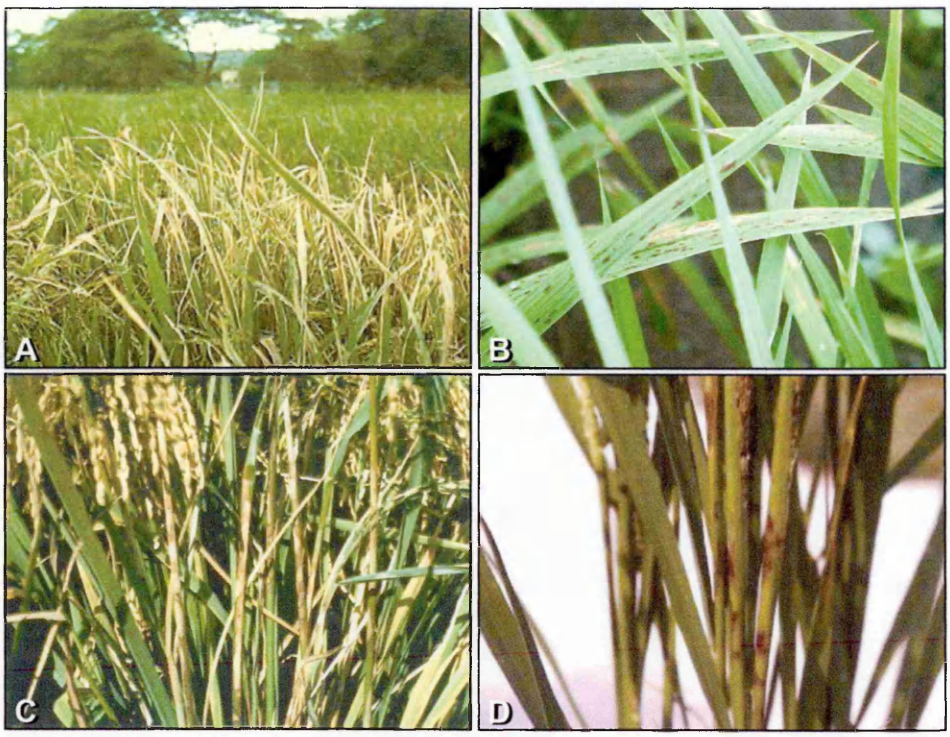


Figure 6: Disease symptoms produced on rice plants by *Xanthomonas oryzae* pv. *oryzae*, causal organism of bacterial blight (A) (http://seedcenter17.doae.go.th/farmer/pest/rice_xx2-05_newDisease009.html), *Magnaporthe grisea*, causal organism of blast (B) (http://beaumont.tamu.edu/Research/Agroecosystems/Rice/RiceBlast.htm), *Rhizoctonia solani*, causal organism of sheath blight (C) (http://www.ricethailand.go.th/rkb/data_005/rice_xx2-05_newDisease005.html) and *Pseudomonas fuscovaginae*, causal organism of sheath brown rot (D) (http://narc.naro.affrc.go.jp/byogai/saikin/subpage/gallery/diseases/youshoukappen.htm).

Xanthomonas oryzae pv. *oryzae* and *Pseudomonas fuscovaginae*, the causal of bacterial blight and sheath brown rot respectively, are the subject of separate studies presented further in this work.

Table 1: Main bacteria, fungi, viruses and nematodes rice pathogens.

Pathogens	Disease
Bacteria	
Acidovorax avenae	Bacterial Brown Stripe
Burkholderia glumae	Grain Rot / Seedling Rot
Burkholderia plantarii	Seedling Blight
Erwinia chrysanthemi	Foot Rot
Pantoea agglomerans	Palea Browning
Pseudomonas fuscovaginae	Sheath Brown Rot
Pseudomonas syringae pv. *syringae*	Sheath Rot
Pseudomonas syringae pv. *oryzae*	Bacterial Halo Blight
Xanthomonas oryzae pv. *oryzae*	Bacterial Blight
=*Xanthomonas campestris* pv. *oryzae*	
Xanthomonas oryzae pv *oryzicola*	Bacterial Leaf Streak
Fungi	
Alternaria padwickii	Stackburn (Alternaria Leaf Spot)
Achlya conspicua	Seed Rot / Seedling Disease
Achlya klebsiana	Seed Rot / Seedling Disease
Bipolaris oryae	Brown Spot / Kernel Spotting /
= *Cochliobolus miyabeanus* (teleomorph)	Seedling Blight
Cercospora jansena = *C. oryzae*	Narrow Brown Leaf Spot
= *Sphaerulina oryzina* (teleomorph)	
Curvularia lunata	Black Kernel
= *Cochiobolus lunatus* (teleomorph)	
Curvularia spp.	Kernel Spotting / Seedling Blight
Drechslera gigantea	Eye Spot
Entyloma oryzae	Leaf Smut
Fusarium spp.	Kernel Spotting / Root Rots /
	Seedling Blight
Gaeumannomyces graminis	Crown sheath rot
Microdochium oryzae	Leaf scald / Kernel Spotting
= *Rhynchosporium oryzae*	
Magnaporthe salvinii	Stem Rot
Pythium spp.	Root Rots / Seed Rot /
	Seedling Disease
Pyricularia grisea = *P. oryzae*	Blast
=*Magnaporthe grisea* (teleomorph)	
Rhizoctonia oryzae-sativae	Aggregate sheath spot / Sheath spot
= *Ceratobasidium oryzae-sativae* (teleomorph)	
Rhizoctonia solani	Seedling Blight / Sheath Blight
= *Thanatephorus cucumeris* (teleomorph)	
Sarocladium oryzae	Kernel Spotting / Sheath Rot
Sclerophthora macrospora	Downy Mildew
Viruses	
Barley Yellow Dwarf	Giallume
Rice Black Streak Dwarf (RGSDV)	Rice Black Streak Dwarf
Rice Dwarf (RDV)	Rice Dwarf
Rice Grassy Stunt (RGSV)	Rice Grassy Stunt
Rice Hoja Blanca (RHBV)	Rice Hoja Blanca
Rice Necrotic Mosaic (RNMV)	Rice Necrotic Mosaic
Rice Rugged Stunt (RRSV)	Rice Rugged Stunt
Rice Stripe (RStV)	Rice Stripe
Rice Transitory Yellowing (RTYV)	Rice Transitory Yellowing
MLO	Rice Yellow Dwarf
Rice Yellow Mottle (RYMV)	Rice Yellow Mottle
Rice Tungro Spherical (RTSV)	Tungro (Rice Tungro Disease - RTD)
Rice Tungro Bacilliform (RTBV)	Tungro (Rice Tungro Disease - RTD)
Nematodes	
Aphelenchoides besseyi	Crimp Nematode
Ditylenchus angustus	Stem Nematode
Hirschmaniella oryzae	Root Nematode
Meloidogyne spp.	Root-Knot

1.6 Xanthomonas oryzae pv. *oryzae*

The present taxonomic status of *Xanthomonas oryzae* pv. *oryzae* (ex Ishiyama 1922) is the result of integrated phenotypic and genotypic analyses (Swings *et al.*, 1990). Earlier classifications were "*Pseudomonas oryzae*" Uyeda and Ishiyama 1926, "*Xanthomonas oryzae*" (Uyeda and Ishiyama 1926) Dowson 1943, and "*Xanthomonas campestris* pv. *oryzae*" (Ishiyama 1922) Dye 1978.

The species *X. oryzae* includes two pathovars: *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, the latter being the causal agent of bacterial leaf streak of rice (Fang *et al.*, 1957).

Traditionally, *X. oryzae* pv. *oryzae* (*Xoo*) populations have been characterized by virulence typing on a set of differential cultivars carrying resistance genes, thus establishing races or pathotypes (Mew, 1987). In the Philippines, ten races of *Xoo* have been defined based on a set of near-rice-isogenic lines (called IRBB lines) carrying 12 individual bacterial blight resistance genes in the common genetic background of IR24. *Xoo* is a vascular pathogen that infects rice plants through hydathodes or wounds (Mew *et al.*, 1984). Upon entering the leaf, the pathogen multiplies in the mesophyll intercellular spaces and gains access to the xylem vessels. The pathogen multiplies rapidly and drops of bacterial ooze on the lesion surface can infect other plants by contact or via water during irrigation. Secondary spread of *Xoo* in tropical region is often associated with occurrence of typhoons, that promote the infection dispersing bacteria and causing wounds (EPPO, 2007).

1.7 Pseudomonas fuscovaginae

Pseudomonas fuscovaginae, a Gram-negative fluorescent pseudomonad, was first reported in Japan (Miyajima *et al.*, 1983; Tanii *et al.*, 1976). Subsequently the pathogen has been isolated from rice in Central America, Latin America and Madagascar (Duveiller *et al.*, 1988; Rott and Notteghem, 1989; Zeigler and Alvarez, 1987). *P. fuscovaginae* was also reported to be pathogenic to other cereals, such as wheat (*Triticum aestivum*), sorghum (*Sorghum bicolor*) and maize (*Zea mays*) (Duveiller *et al.*, 1989; Duveiller, 1990), as well as a broad range among wild grasses (Miyajima *et al.*, 1983). Isolation and identification of the pathogen is not often easy because various fluorescent pseudomonads, most of them saprophytes, can be isolated from rice sheath rot. The combination of biochemical tests, serological technique and pathogenicity tests are necessary for identification (Rott, 1991). *P. fuscovaginae* favors cool temperatures (15-23°C) and high humidity for disease development at the booting stage (Miyajima *et al.*, 1983). The cool night temperatures and high rainfall in humid tropical highlands might favor the infection. The pathogen is seedborne and considered seed-transmitted. One measure to control the infection is seed treatment by heat therapy at 65 °C for six days (Zeigler and Alvarez, 1987). Symptoms commonly include brown necrotic lesions on the flag leaf sheath or extensive necrosis of the sheath, with grain discoloration and sterility in severe cases (Duveiller *et al.*, 1989; Miyajima *et al.*, 1983; Zeigler and Alvarez, 1987). Disease development and symptoms progression are believed to be associated with the production of several phytotoxins (Gross and Cody, 1985; Gross, 1991).

Despite the importance of *P. fuscovaginae* as an opportunistic pathogen on several plant hosts around the world, no molecular studies of virulence have thus far been reported.

1.8 Signalling in plant-microbe interactions

For many years the purpose of microbiology was to understand the behaviour of model microbes under laboratory conditions. The challenge for the future is to understand the behaviour of model microbes in their natural habitats. Based on where they live, microbes interacting with plants can be found in the rhizosphere, surrounding the roots, or in the phyllosphere (the total above-ground surfaces of a plant). Based on their effect on the plant, microbes can be classified as pathogenic, saprophytic and beneficial. Pathogens can infect leaves, stems and roots; saprophytes live on dead plant material; beneficial microbes help the plant to obtain water and nutrients and protect the plant from pests and pathogens (Lugtenberg *et al.*, 2002).

More than 100 genes are required for bacterial pathogenicity, they can be classified into three groups: pathogenicity genes, virulence genes, and host range genes. Pathogenicity genes are bacterial genes needed for growth on or in plants. One example are the *hrp* genes (*h*ypersensitive *r*esponse and *p*athogenicity), involved in the production of signal molecules, which elicit defence responses associated with hypersensitivity in non-host plants (Lugtenberg *et al.*, 2002). Virulence genes contribute to the aggressiveness of the pathogen and are necessary for symptom production in the host plant. General virulence factors include toxins, extracellular polysaccharides (EPS), plant growth hormones and enzymes (e.g. proteases, cellulases, pectic enzymes, cell-wall degrading enzymes). Host range genes determine the plant

species and the cultivars that can be infected by factors that act positively (specific virulence factors) or negatively (avirulence genes - *Avr*) (Lugtenberg *et al.*, 2002).

The interaction between pathogenic bacteria and the host plant can be classified into two general categories: compatible interaction and incompatible interaction. Compatible interaction occurs between virulent pathogens and susceptible host plants, resulting in the development of disease symptoms. Incompatible interaction results in little or no disease symptoms since the bacterium induces a defence response in the plant (Kim *et al.*, 2008). Usually incompatible reactions are characterized by a hypersensitive response (HR) of the resistant plant, which refers to localized cell death that occurs rapidly at the site of bacterial invasion (Lugtenberg *et al.*, 2002).

1.8.1 Plant recognition by microbes

Successful infection of a plant by pathogens requires mechanisms of attachment, invasion and inactivation of plant defence. Plants have developed a sophisticated immune system to defend themselves. However, unlike animals, that have an adaptive immune system, plants have to rely to their innate immunity to react against most potential pathogens.

Recognition is considered the first event to trigger the plant immune response. It can occur either through the detection of many common pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) found in the host cells (He *et al.*, 2007), or through the cultivar-specific resistance, involving effector molecules from the pathogen and the corresponding resistance (R) proteins in the plant (Ingle *et al.*, 2006).

In the first case, the perception of different PAMPs/MAMPs occurs through specific pattern-recognition receptors (PRRs) that induce the defence response in the host plant, activating plant immunity (He *et al.*, 2007; Jones and Dangl, 2006). In the second case, it occurs using the polymorphic NB-LRR protein products, encoded by the R genes, having the characteristic nucleotide binding (NB) and leucine rich repeat (LRR) domains (Jones and Dangl, 2006).

Examples of PAMPs, identified in Gram-negative bacteria plant pathogens, are flagellin, cold-shock protein, lipopolysaccharide (LPS) and elongation factor Tu (EF-Tu) (He *et al.*, 2007; Jones and Dangl, 2006). These molecules typically contain a short (10-25) amino acid epitope that elicit a stronger defence response than the complete protein (Ingle *et al.*, 2006). In *Arabidopsis thaliana*, the flagellin receptor FSL2 recognizes fgl22, a 22 amino acid conserved peptide of bacterial flagellin, sufficient to trigger the plant innate immune response (He *et al.*, 2007; Ingle *et al.*, 2006; Jones and Dangl, 2006; Kim *et al.*, 2008).

Lipopolysaccharide (LPS), the principal component of the outer membrane of Gram-negative bacteria, contains a long-chain polysaccharide, highly variable, termed O-antigen and a highly conserved part constitute by the oligosaccharide core and the lipid A. This invariable part of the outer membrane is the most potent stimulator of innate immunity and is considered as a prototypic model of PAMP (Zipfel and Felix, 2005). The first 18 amino acids of the elongation factor EF-Tu, termed elf18, from *E. coli* and other bacteria, has been found to function as a MAMP in *Arabidopsis thaliana*, binding the EF-Tu receptor (EFR) (Kim *et al.*, 2008; Zipfel and Felix, 2005).

In mammals, PAMPs/MAMPs are recognized by several structurally different PRRs, the most prominent group comprises the Toll-like receptors (TLRs), a family of transmenbrane proteins containing leucine-rich repeat (LRR) domains that sense bacteria, fungi, protozoa and viruses. Plants appear not to have clear homologs of TLRs, but they have large gene-families that encode receptor-like kinases (RLKs) and receptor-like proteins (RLPs) (Zipfel and Felix, 2005).

Typically RLKs contain a signal sequence, a transmembrane region, and a C-terminal domain. The plant RLKs likely are transmembrane proteins that perceive signals through their extracellular domains and propagate the signals via their intracellular kinase domains (van der Geer *et al.*, 1994). The kinase domains of plant RLKs belong to the same gene family as those of *Drosophila melanogaster* Pelle and mammalian interleukin receptor-associated kinases (Shiu and Bleecker, 2001). The biological functions of plant RLK/Pelle family members can be classified into two broad categories (Shiu and Bleecker, 2003). The first category includes RLKs that control plant growth and development (Becraft, 2002). The second category includes RLKs involved in plant-microbe interactions and defence responses. In this category, some RLKs are involved in plant-pathogen interactions, such as rice (*Oryza sativa*) Xa21 in resistance to bacterial pathogen (Song *et al.*, 1995).

The early immune response downstream MAMP recognition involve the activation of conserved mitogen-activated protein kinase (MAPK) signalling cascade. A MAPK cascade usually involves a MAPKKK(MTK)-MAPKK(MKK)-MAPK(MPK) module that transduces extracellular signals through the receptors into the cell (He *et al.*, 2007; Ingle *et al.*, 2006).

In plants, different PAMPs/MAMPs activate defence responses such as changes in cytoplasmatic Ca^{2+} levels, activaton of MAPK cascades, induction of defence-related genes, reactive oxygen species (ROS) and nitric oxide (NO) (He *et al.*, 2007; Ingle *et al.*, 2006).

Type III secretion system (TTSS), present in both animal and plant pathogenic bacteria, is a key virulence determinant used by bacteria to deliver effector proteins directly into the host cell cytoplasm (Galan and Collmer, 1999). It is like a molecular syringe through which a bacterium can inject proteins into eukaryotic cells. Made up of more than 20 proteins, TTSS has three distinguishing features: the absence in the secreted proteins of a cleavable signal peptide, the requirement for customized accessory proteins (chaperones) for many of the secreted proteins, and a widespread requirement for host cell contact for full activation of the secretory pathway (Galan and Collmer, 1999). Bacterial effectors contribute to pathogen virulence by mimicking or inhibiting eukaryotic cellular functions (Jones and Dangl, 2006).

The type III secretion system (TTSS) in *Xanthomonas campestris* pv. *vesicatoria* is necessary for bacterial pathogenicity in susceptible hosts. In addition this pathogen produces filamentous structures, Hrp pili, that are in close contact with the TTSS during the delivery of type III effector proteins to the host plant (Weber *et al.*, 2005).

Agrobacterium tumefaciens can genetically transform numerous plant and fungal species by injecting a single stranded T-DNA (transferred DNA) into the host cell using a type IV secretion system (T4SS). The T4SS of *Agrobacterium* is encoded by 11 *virB* genes and *virD4*, in the virulence (*vir*) region of the Ti-plasmid (tumor-inducing plasmid), that form two functional components: a filamentous *pilus* and

a membrane-associated transporter complex (McCullen and Binns, 2006). Rhizobia are able to invade roots, leading to the formation of nodules, in a complex process that require a continuous signal exchange between plant and bacteria. Flavonoids activate the expression of the bacterial nodulation (*nod*) genes involved in the synthesis and secretions of Nod-factors (NF) recognized by the plant. These factors together with additional microbial signal molecules, such as polysaccharides and secreted proteins, allow bacteria attached to root hairs to penetrate the roots (Soto *et al.*, 2006). This section highlights some of the very complex interactions between plants and bacteria; this is probably a result of co-evolution for a very long period of time.

1.8.2 Plant colonization by microbes

Colonization of the plant tissue is a crucial step in pathogenesis as well as for beneficial effects of microbes on the plant. Microbial success depends critically on the ability to perceive and respond rapidly to changes in the environment. The plant surface contains many microbes which are not homogeneously distributed; such microcolonies are ideal places for bacteria to communicate with each other. In complex ecosystems they must simultaneously exchange signals with members of their own species as well as members of other species of microorganisms and eukaryotes. In this context, a small molecule generated by one organism is sensed by another, leading to a response that usually involves a change in gene expression. The ability to regulate gene expression in response to changes in population density is a process known as quorum sensing (QS) (Bassler, 1999). QS is mediated by small, diffusible signal molecule often called autoinducers which accumulate as the

bacterial population increases. Several chemical classes of microbially-derived signalling molecules have been identified; these can be generally divided in two main categories: amino acids and short peptides are commonly used by Gram-positive bacteria, whereas fatty acid derivatives are most often utilized by Gram-negative bacteria (Miller and Bassler, 2001).

1.9 Quorum Sensing in Gram-negative bacteria

Quorum Sensing and autoinduction was first described in two luminous marine bacterial symbionts: *Vibrio fischeri* and *Vibrio harveyi* (Nealson *et al.*, 1970). *V. fischeri* colonizes the light organ of marine fishes and squids, reaching high cell densities (10^{10}cells/ml) and producing bioluminescence. The enzymes responsible for light emission are encoded by the luciferase structural operon *luxCDABE* only at high cell density in response to the accumulation of secreted signal molecules (Nealson and Markovitz, 1970). These secreted molecules were shown to be 3-oxo-*N*-(tetrahydro-2-oxo-3-furanyl)-hexanamide, commonly known as *N* -3-(oxohexanoyl)-homoserine lactone (3-oxo-C_6-AHL) (Eberhard *et al.*, 1981).

In *V. fischeri* 3-oxo-C_6-AHL is synthesized via the LuxI protein, an AHL synthase, that catalyzes the formation of an amide bond between S-adenosylmethionine (SAM) and an acylated acyl carrier protein (ACP) and then catalyzes the formation of the acyl homoserine lactone from the acyl-SAM intermediate (Figure 7) (Schaefer *et al.*, 1996). *N*-AHL signal molecules reach a threshold or "quorum" level at high cell density and are recognized by the cognate LuxR protein, forming an active complex for regulation of target genes. The LuxR protein consists of two functional domains: the amino-

terminal domain, involved in AHL binding, and the carboxyl-terminal domain, required for DNA binding and transcriptional regulation (Slock *et al.*, 1990).

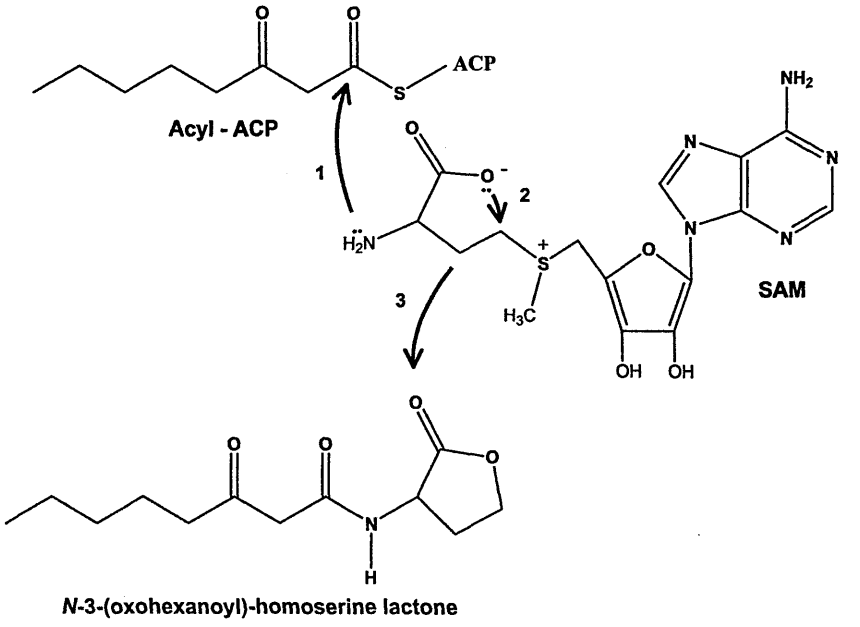


Figure 7: LuxI-directed biosynthesis of *N*-3-(oxohexanoyl)-homoserine lactone by the amide linkage between S-adenosylmethionine (SAM) and an acylated acyl carrier protein (ACP) in *V. fischeri* (Miller and Bassler, 2001).

One target promoter is upstream of the gene encoding the synthase *luxI* gene, thus creating a positive feedback circuit in the quorum system. Each eukaryotic host uses the light provided by the bacteria for a specific purpose: illumination enable squid to avoid predators during clear nights, when moonlight penetrates the seawater and they would produce shadows; in contrast the knight-fish *Monocentris japonicus* uses bacterial light to attract a mate. Even if purposes are different, the regulation of light production in the specialized light organs is identical; light emission is tightly

correlated with the cell-population density of the bacterial culture in the organ (Miller and Bassler, 2001). The structure of AHLs produced by different bacteria is highly conserved, consisting of a homoserine lactone ring connected to a fatty acyl chain. Many AHL signal molecules have been characterized so far, differing in acyl chain length (usually 4-12 carbons) and substitution at the third carbon of the chain (Figure 8). Most AHLs are believed to freely diffuse across the cell wall, with the exception of long-chain AHLs that utilize an efflux pump for translocation across the cell membrane (Pearson *et al.*, 1999).

In Gram-negative bacteria, AHL-mediated cell-cell signalling plays a role in regulating important bacterial functions such as antibiotic biosynthesis, production of virulence factors, exopolysaccharide formation, biofilm development, bacterial motility and plasmid conjugal transfer (Miller and Bassler, 2001).

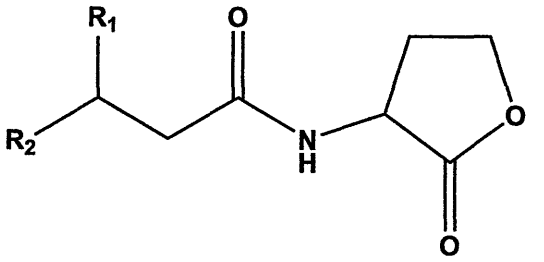


Figure 8: General structure of a *N*-Acyl homoserine lactone (AHL) molecule. R_1 can be: –H, =O or –OH, generating an unsubstituted, an oxo- or an hydroxy-AHL respectively, while R_2 can be: – CH_3, – $(CH_2)_{2\text{-}14}CH_3$ or –$(CH_2)_5CH{=}CH(CH_2)_5CH_3$, generating differences in acyl chain length (Soto *et al.*, 2006).

1.10 Purification and characterization of AHL QS signal molecules

Normally QS molecules are efficiently extracted from cell-free supernatant using dichloromethane, chloroform or ethyl acetate in acidified media; the last method is

usually applied to extract AHLs. Autoinducers can be subsequently separated by HPLC (High Performance Liquid Chromatography), using a methanol or an acetonitrile gradient, or by a C_{18} reversed-phase TLC (Thin-Layer Chromatography) and developed with a methanol/water (60:40 vol/vol) mobile phase. The TLC plate is loaded with the sample to test and with different synthetic AHLs as standards and, after chromatography, is overlaid with a soft agar suspension containing the most suitable bacterial biosensor (Shaw *et al.*, 1997). Many biosensors have been developed so far; they do not produce AHLs and contain a functional LuxR-family protein cloned with the cognate *luxI* target promoter, that positively regulates the transcription of a reporter gene (e.g. bioluminescence, β-galactosidase, green-fluorescent protein and violacein pigment production) (Steindler and Venturi, 2007). Depending on the LuxR-family protein, each biosensor is able to specifically recognize only a few AHL molecules, therefore sometimes more than one biosensor is required to detect all AHLs produced by the strains tested. TLC is not a technique which can tentatively assign structures; these can only be unequivocally determined on the basis of spectroscopic properties, such as mass spectrometry (MS) and nuclear magnetic resonance spectroscopy (NMR).

1.11 QS in plant pathogenic bacteria

Many studies have demonstrated that QS has an important role in plant-pathogen interaction, controlling secondary metabolite production and virulence gene expression. *A. tumefaciens* is a plant pathogen able to induce crown gall tumors in plants by transferring oncogenic DNA from its tumor-inducing Ti plasmid into the chromosome of the plant cells. Conjugation is regulated by two different signalling

mechanisms: one involving conjugal opines, produced by the crown gall tumor, that regulate the expression of the *tra* genes. TraI synthesises 3-oxo-C_8-AHL to stimulate conjugation; TraR/3-oxo-C_8-AHL regulates expression of the *tra* regulon as well as the *traI* promoter, creating a positive feedback loop (Hwang *et al.*, 1994). The *A. tumefaciens* opine and QS signal pathways are linked to one another in a hierarchical fashion, with opines being the dominant regulator.

Many species of *Erwinia* have been found to produce AHLs. The pathogenicity of *Erwinia carotovora*, the causative agent of plant soft rots and the potato disease blackleg, is related to the production of various plant tissue-degrading enzymes that are involved in the maceration of the plant tissue for microbial colonization of the host. *E. carotovora* employs the ExpI/ExpR QS system, based on the 3-oxo-C_6-AHL signal molecule, to ensure that exoenzymes production does not occur until sufficient bacterial numbers have been achieved (Pirhonen *et al.*, 1993). A second QS system exists in the *E. carotovora* genome, the CarI/CarR circuit, that acts using the same AHL molecule and regulates the production of a carbapenem antibiotic (McGowan *et al.*, 1995). It appears that *E. carotovora* has developed a sophisticated strategy to counteract the competing microflora by coordinating the production of the carbapenem with the tissue-macerating enzymes. In the closely related *Erwinia chrysanthemi*, the ExpI/ExpR QS system produces and responds to 3-oxo-C_6-AHL and C_6-AHL and is involved in the regulation of pectinase synthesis (Nasser *et al.*, 1998; Reverchon *et al.*, 1998).

The plant-pathogenic bacterium *Erwinia stewartii* (*Pantoea stewartii* subsp. *stewartii*) is the causative agent of Stewart's wilt in sweetcorn and leaf blight in maize. The QS

system of this microorganism consists of the EsaI/EsaR, which are LuxI/LuxR homologues respectively, and 3-oxo-C_6-AHL is required for the cell density-dependent production of an extracellular heteropolysaccharide (EPS) capsule which plays several roles in disease development (Beck von Bodman and Farrand, 1995).

Pseudomonas aeruginosa can be both, an animal and a plant pathogen: in humans it is responsible for infection in immune-compromised individuals or in cystic fibrosis patients, whereas in many plants it is an opportunistic pathogen that causes soft rot. *P. aeruginosa* has two AHL QS systems, the LasI/LasR and the RhlI/RhlR. The first one is responsible for production and regulation via 3-oxo-C_{12}-AHL, while RhlI catalyzes the synthesis of C_4-AHL, afterwards recognized by RhlR (Pearson *et al.*, 1995). Both LasR and RhlR, along with their cognate AHLs, affect the expression of extracellular virulence factors and secondary metabolites that contribute to the growth of bacteria *in planta* (Miller and Bassler, 2001; Rahme *et al.*, 2000).

R. solanacearum causes a vascular wilt disease in more than 200 plant species, including tobacco, tomato, potato, peanut and bananas. This pathogen causes wilt mainly through the production of a high-molecular-mass acidic extracellular polysaccharide (EPS I) which can occlude vascular tissue and prevent water flow (Flavier *et al.*, 1997b).

Virulence in *R. solanacearum* is controlled by the 3-OH-PAME signal molecule that regulates virulence factors through the LysR-type transcriptional regulator PhcA (see also paragraph *1.13*). A classical QS system, SolI/SolR, has been found in *R. solanacearum* producing C_6- and C_8-AHLs, although it was not directly involved in virulence, but was found to be controlled by PhcA (Flavier *et al.*, 1997a).

Burkholderia glumae causes rice grain rot and seedling rot in rice producing a broad-host range phytotoxin called toxoflavin. Two QS protein, TofI/TofR, were found to be responsible for the production and the detection of the two AHL signal molecules, identified as C_6- and C_8-AHL. The *tofI* mutant failed to produce toxoflavin (Kim *et al.*, 2004) and the C_8-AHL-TofR complex was found to regulate a lipase involved in rice pathogenicity (Devescovi *et al.*, 2007), indicating that QS plays a pivotal role in *B. glumae* virulence.

Table 2: Summary of quorum sensing systems in plant pathogenic bacteria.

Organism	Major signal molecule	Regulatory proteins	Phenotype	Reference
Agrobacterium tumefaciens	3-oxo-C_8-AHL	TraI/TraR	Ti plasmid conjugation	(Hwang *et al.*, 1994)
Burkholderia glumae	C_8-AHL C_6-AHL	TofI/TofR	Toxoflavin regulation Lipase	(Kim *et al.*, 2004) (Devescovi *et al.*, 2007)
Erwinia carotovora	3-oxo-C_6-AHL	ExpI/ExpR CarI/CarR	Exoenzymes production Carbapenem production	(Pirhonen *et al.*, 1993) (McGowan *et al.*, 1995)
Erwinia chrysantemi	3-oxo-C_6-AHL C_6-AHL	ExpI/ExpR	Pectate lyases	(Nasser *et al.*, 1998) (Reverchon *et al.*, 1998)
Erwinia stewartii	3-oxo-C_6-AHL	EsaI/EsaR	Capsular polysaccharide Virulence factors	(Beck von Bodman and Farrand, 1995)
Pseudomonas aeruginosa	3-oxo-C_{12}-AHL C_4-AHL	LasI/LasR RhlI/RhlR	Exoprotease virulence factors Biofilm formation	(Miller and Bassler, 2001 and references therein)
Ralstonia solanacearum	C_6-AHL C_8-AHL	SolI/SolR	Unknown	(Flavier *et al.*, 1997b)

1.12 Non-AHL QS molecules in plant-microbe interaction

Several bacterial species that interact with plants have been shown to produce AHLs or other signalling compounds. A further signalling molecule, apart from 3-oxo-C_{12}-AHL and C_4-AHL, has been identified in *P. aeruginosa*, a 2-heptyl-3-hydroxy-4-quinolone,

designated as the *Pseudomonas* quinolone signal (PQS) (Figure 9), dependent on the QS system for its production and bioactivity (Pesci *et al.*, 1999). In addition two cyclic dipeptides in the cell-free supernatant of *P. aeruginosa* culture were found to be able to activate an AHL biosensor. The molecules were identified as diketopiperazines (DKPs): cyclo(ΔAla-L-Val) and cyclo(L-Pro-L-Tyr) (Figure 9). These two DKPs and a third cyclic dipeptide, a cyclo(L-Phe-L-Pro), isolated and characterized from *Pseudomonas fluorescens* and *Pseudomonas alcaligenes,* can activate the QS system and can compete for the same LuxR-binding site (Holden *et al.*, 1999).

In *Bradyrhizobium japonicum*, a symbiotic microorganism, a novel cell density factor (CDF) was found to mediate the repression of the nodulation genes in an iron-dependent manner. This molecule has been characterized as 2-{ -[[4-(3-aminooxetan-2-yl) phenyl]-(imino) methyl] phenyl} oxetan-3- ylamine, named in a simple manner bradyoxetin (Loh *et al.*, 2002). A volatile extracellular factor (VEF) produced by *R. solanacearum*, involved in regulation of virulence genes, was purified from spent culture supernatant and identified as 3-hydroxypalmitic acid methyl ester (3-OH PAME) (Figure 9) (Flavier *et al.*, 1997a). PhcB is essential for the production of 3-OH PAME that acts as an exponential-phase signal that regulates, through the PhcS/PhcR two component system, PhcA, a LysR-type transcriptional regulator. PchA then regulates exopolysaccharides (EPS) and plant cell wall degrading enzymes which are important factors for colonization of host tissues (Clough *et al.*, 1997). Two diffusible signalling molecules are related to pathogenicity in *Xanbthomonas campestris* pv. *campestris* (*Xcc*): the diffusible signal factor (DSF) and the diffusible factor (DF) (Figure 9).

Xcc is a vascular pathogen of cruciferous plants, it multiplies in the xylem eventually blocking the vessel with bacterial cells and xanthan gum, which is the major EPS produced by xanthomonads. Most of the pathogenicity genes, including the *gum* genes, are coordinately regulated by the Rpf (Regulation of Pathogenicity Factors) system which carries nine *rpf* genes (*rpfA-I*). Among them, *rpfB* and *rpfF* are involved in DSF production, a fatty acid derivative that regulates extracellular enzymes, EPS and cyclic glucans (Barber *et al.*, 1997; Vojnov *et al.*, 2001). DF is a butyrolactone, the locus implicated in the synthesis of DF in *Xcc* strain B-24 is *pigB*, mutations in which cause a reduction in levels of EPS and xanthomonadin pigment production (Poplawsky and Chun, 1997).

A second QS system, termed LuxS/LuxPQ, has been identified in *Vibrio harveyi*. LuxS synthesizes the signal molecule AI-2, which has been characterized as a furanosyl borate diester (Figure 9). LuxP is a periplasmic-binding protein that binds AI-2. The complex is afterwards detected by LuxQ, an inner membrane sensor kinase. At high cell density, upon ligand binding, LuxQ switches from kinase to phosphatase, removing the phosphate from the response regulator LuxO, via the intermediate protein LuxU. Unphosphorylated LuxO is inactivate and cannot promote the expression of sRNAs (small regulatory RNAs), required for destabilization of mRNA encoding the activator protein LuxR, that is so expressed and can bind the *luxCDABE* operon, producing bioluminescence. LuxS homologues have been found in numerous Gram-negative and Gram-positive organisms, producing compounds capable of activating AI-2 biosensors (Henke and Bassler, 2004; Xavier and Bassler, 2003).

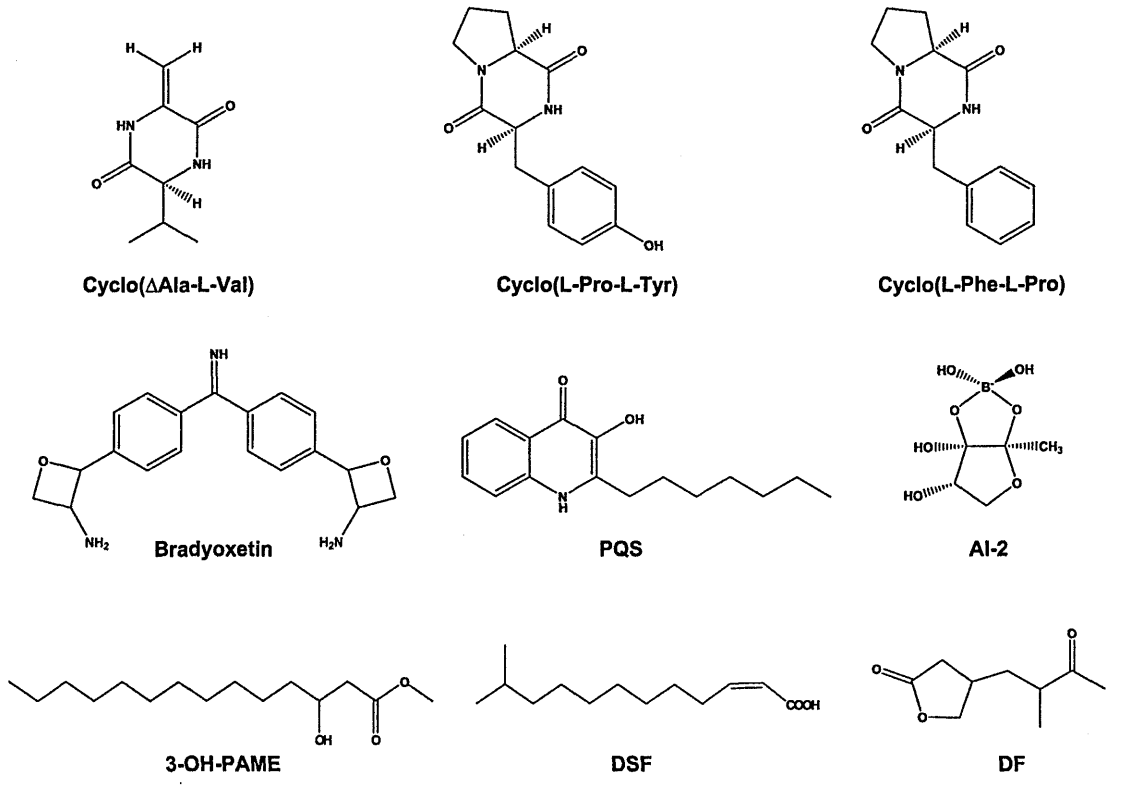


Figure 9: Non-AHL signal molecules: cyclic dipeptides in *P. aeruginosa* and *P.fluorescens*, cyclo(ΔAla-L-Val) and cyclo(L-Pro-L-Tyr) and cyclo(L-Phe-L-Pro); bradyoxetin in *Bradyrhizobium japonicum*, PQS in *P. aeruginosa*; AI-2 in *Vibrio harveyi*; 3-OH-PAME in *Ralstonia solanacearum*; DSF and DF in *Xathomonas campestris* pv. *campestris* (Holden *et al.*, 1999; Soto *et al.*, 2006; Xavier and Bassler, 2003).

1.13 LuxR-family orphans

In some bacterial species a quorum sensing-like LuxR-homologue has been found and termed a LuxR-family orphan due to the absence of the cognate LuxI-homologue. The presence of LuxR-family orphans has been reported for species having a functional AHL QS system, as well as species in which no AHL QS system has been found.

The wild-type *Sinorhizobium meliloti*, strain Rm1021, possesses at least two QS systems: the MelI/R system, that controls the synthesis of short-chain AHLs, involved

in nodulation and invasion; and the SinI/R system, responsible for the synthesis of long-chain AHLs, that activates the expression of *exp* genes for exopolysaccharide production. The expression of the *exp* genes also requires the presence of an additional regulator, ExpR, an orphan LuxR-homologue. ExpR plays a role in activation of EPSII production, motility, nitrogen fixation and transport of small molecules together with the Sin QS-system (Hoang *et al.*, 2004).

A similar scenario has been described in *P. aeruginosa*, in which a third LuxR-homologue is present in addition to the LasI/R and RhlI/R QS systems. This LuxR homologue is an orphan since it is devoid of the cognate LuxI-family protein and has been designated QscR (quorum-sensing-control repressor). QscR is immediately upstream of the *phz* operon (*phz*A2-G2) responsible for phenazine pigment production. Null mutants in *qscR* have an hypervirulence phenotype and form blue-pigmented colonies, indicative of phenazine overproduction. Moreover *lasI* and *rhlI* are both prematurely transcribed also resulting in the premature production of the AHL signals, 3-oxo-C_{12}-AHL and C_4-AHL, indicating that QscR acts as a repressor (Chugani *et al.*, 2001).

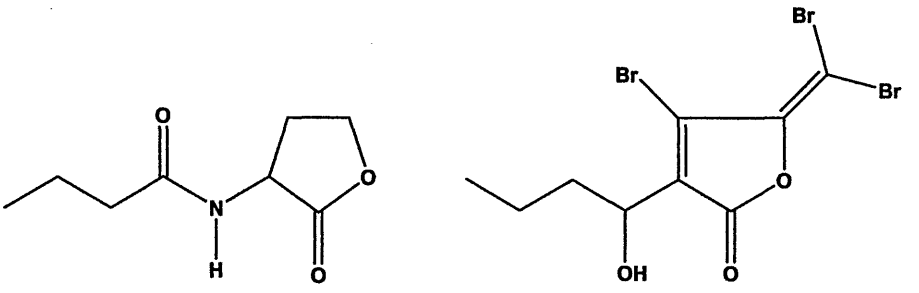
An orphan LuxR-homologue, SdiA, has also been described in *Escherichia*, *Salmonella* and *Klebsiella* spp.; these bacteria do not produce AHLs. In *S. enterica* serovar Typhimurium the putative AHL receptor SdiA, can respond to 3-oxo-C_6-AHL and 3-oxo-C_8-AHL exogenously provided in a semisolid medium, leading to the hypothesis that SdiA is used to detect signals produced by other bacteria species (Michael *et al.*, 2001).

1.14 Plant signal molecules involved in bacterial regulation

Among higher plants AHL mimic molecules have been identified demonstrating that plants can secrete compounds that affect bacterial QS signalling and regulation.

In the marine red alga *Delisea pulchra*, halogenated furanones, which are structurally similar to AHL molecules (Figure 10), have many biological activities, including antimicrobial properties. Swarming motility in *Serratia liquefaciens*, regulated by QS, was affected by the presence of *D. pulchra* furanones reducing the velocity of spreading over the surface, while swimming motility, a QS independent process, was not affected (Givskov *et al.*, 1996). Further studies demonstrated that furanones can control marine bacterial colonization by binding competitively to the LuxR homologue protein (Manefield *et al.*, 1999).

By contrast to the *D. pulchra* furanones, which have an inhibitory function, in the unicellular green alga *Chlamydomonas reinhardtii* AHL mimics can stimulate gene expression of specific AHL receptors (Teplitski *et al.*, 2004). Also higher plants, such as pea, rice, soybean, tomato were shown to produce molecules that appear to be recognized by LuxR homologue proteins, and have specific effects on QS-regulated behaviours in bacteria (Daniels *et al.*, 2002; Teplitski *et al.*, 2000). Many different biosensors, based on different signal molecule specificity, have been used to study methanol and ethyl acetate extracts from the legume *Medicago truncatula*, indicating the presence of more than a dozen of active molecules having both stimulatory or inhibitory effects on the sensor protein (Gao *et al.*, 2003).



N-butanoyl-homoserine lactone (C_4-AHL) Halogenated furanone

Figure 10: Structural similarity between the typical AHL molecule and the halogenated furanone molecule from *Delisea pulchra* (Bauer and Mathesius, 2004).

Similarly, ethyl acetate extract from rice plants was able to positively stimulate specific AHL biosensors and was found to be very sensitive to AHL AiiA-lactonase degradation activity (Degrassi *et al.*, 2007). All this data indicates that plants synthesize signal molecules able to interact and interfere with the bacterial QS system. On the other hand, are plants able to perceive and react to the bacterial signals?

In a proteomic study performed on *M. truncatula* it has been demonstrated that nanomolar concentrations of AHLs can affect the accumulation of more than 6% of the proteins recovered from roots (Mathesius *et al.*, 2003).

The ability of plants to detect bacterial signal molecules and elicit systemic responses could be a sophisticated part of the long-evolved adaptations of eukaryotic hosts to interaction with bacteria.

1.15 Aim of the thesis

Quorum sensing in bacteria is often involved in many important processes such as the regulation of virulence factors, the biosynthesis of lytic enzymes or exopolysaccharide production. Bacteria that use QS to regulate gene expression usually produce and secrete small signal molecules which can then be recognized by other bacteria of the same species and possibly by bacteria of different species, thus creating a complex communication network. In addition, some bacteria possess more than one QS system or may possess orphan LuxR-family regulatory proteins able to detect a signal molecule(s).

In this study we analysed the QS system of two important rice pathogens, *Xanthomonas oryzae* pv. *oryzae* and *Pseudomonas fuscovaginae*, both responsible for important losses in rice harvesting and both able to enter the plant through the leaves. Despite their importance, very few molecular studies on QS in these pathogens have been performed. Many strains of both species, isolated from different countries world-wide, have been studied for their AHLs production, presence and role of the QS system in rice virulence and colonization.

1.16 REFERENCES

Agarwal, V.K., and Sinclair, J.B. (1997) Principles of Seed Pathology, 2nd Edition. *CRC Press, New York.*

Barber, C.E., Tang, J.L., Feng, J.X., Pan, M.Q., Wilson, T.J., Slater, H., Dow, J.M., Williams, P., and Daniels, M.J. (1997) A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol Microbiol* **24**: 555-566.

Bassler, B.L. (1999) How bacteria talk to each other: regulation of gene expression by quorum sensing. *Curr Opin Microbiol* **2**: 582-587.

Bauer, W.D., and Mathesius, U. (2004) Plant responses to bacterial quorum sensing signals. *Curr Opin Plant Biol* **7**: 429-433.

Beck von Bodman, S., and Farrand, S.K. (1995) Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an *N*-acylhomoserine lactone autoinducer. *J Bacteriol* **177**: 5000-5008.

Becraft, P.W. (2002) Receptor kinase signaling in plant development. *Annu Rev Cell Dev Biol* **18**: 163-192.

Chugani, S.A., Whiteley, M., Lee, K.M., D'Argenio, D., Manoil, C., and Greenberg, E.P. (2001) QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa. Proc Natl Acad Sci U S A* **98**: 2752-2757.

Clough, S.J., Flavier, A.B., Schell, M.A., and Denny, T.P. (1997) Differential Expression of Virulence Genes and Motility in *Ralstonia* (*Pseudomonas*) *solanacearum* during Exponential Growth. *Appl Environ Microbiol* **63**: 844-850.

Cottyn, B. (2003) Bacteria associated with rice seed from Philippine farmers' fields. PhD Thesis. Gent University. Belgium.

Daniels, R., De Vos, D.E., Desair, J., Raedschelders, G., Luyten, E., Rosemeyer, V., Verreth, C., Schoeters, E., Vanderleyden, J., and Michiels, J. (2002) The *cin* quorum sensing locus of *Rhizobium etli* CNPAF512 affects growth and symbiotic nitrogen fixation. *J Biol Chem* **277**: 462-468.

Degrassi, G., Devescovi, G., Solis, R., Steindler, L., and Venturi, V. (2007) *Oryza sativa* rice plants contain molecules that activate different quorum-sensing *N*-acyl homoserine lactone biosensors and are sensitive to the specific AiiA lactonase. *FEMS Microbiol Lett* **269**: 213-220.

Devescovi, G., Bigirimana, J., Degrassi, G., Cabrio, L., LiPuma, J.J., Kim, J., Hwang, I., and Venturi, V. (2007) Involvement of a quorum-sensing-regulated lipase secreted by a clinical isolate of *Burkholderia glumae* in severe disease symptoms in rice. *Appl Environ Microbiol* **73**: 4950-4958.

Duveiller, E., Miyajima, K., Snacken, F., Autrique, A., and Maraite, H. (1988) Characterization of *Pseudomonas fuscovaginae* and Differentiation from Other Fluorescent Pseudomonads Occurring on Rice in Burundi. *J. Phytopathology* **122**: 97-107.

Duveiller, E., Snacken, F., and Maraite, H. (1989) First detection of *Pseudomonas fuscovaginae* on Maize and Sorghum in Burundi. *Plant Disease* **73**: 514-517.

Duveiller, E. (1990) Bacterial Sheath Rot of Wheat Caused by *Pseudomonas fuscovaginae* in the Highlands of Mexico. *Plant Disease* **74**: 932-935.

Eberhard, A., Burlingame, A.L., Eberhard, C., Kenyon, G.L., Nealson, K.H., and Oppenheimer, N.J. (1981) Structural identification of autoinducer of *Photobacterium fischeri* luciferase. *Biochemistry* **20**: 2444-2449.

EPPO, B. (2007) *Xanthomonas oryzae* **37**: 543-553.

Evenson, R.E., and Gollin, D. (2003) Assessing the impact of the green revolution, 1960 to 2000. *Science* **300**: 758-762.

Fang, C.T., Ren, H.C., Chen, T.Y., Chu, Y.K., Faan, H.C., and Wu, S.C. (1957) A comparison of the rice leaf blight organism with the bacterial leaf streak organism of rice and *Leersia hexandra* Swartz. *Acta Phytopathol. Sin.* **3**: 99-124.

Flavier, A.B., Clough, S.J., Schell, M.A., and Denny, T.P. (1997a) Identification of 3-hydroxypalmitic acid methyl ester as a novel autoregulator controlling virulence in *Ralstonia solanacearum*. *Mol Microbiol* **26**: 251-259.

Flavier, A.B., Ganova-Raeva, L.M., Schell, M.A., and Denny, T.P. (1997b) Hierarchical autoinduction in *Ralstonia solanacearum*: control of acyl-homoserine lactone production by a novel autoregulatory system responsive to 3-hydroxypalmitic acid methyl ester. *J Bacteriol* **179**: 7089-7097.

Galan, J.E., and Collmer, A. (1999) Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284**: 1322-1328.

Gao, M., Teplitski, M., Robinson, J.B., and Bauer, W.D. (2003) Production of substances by *Medicago truncatula* that affect bacterial quorum sensing. *Mol Plant Microbe Interact* **16**: 827-834.

Givskov, M., de Nys, R., Manefield, M., Gram, L., Maximilien, R., Eberl, L., Molin, S., Steinberg, P.D., and Kjelleberg, S. (1996) Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. *J Bacteriol* **178**: 6618-6622.

Gross, D.C., and Cody, Y.S. (1985) Mechanisms of plant pathogenesis by *Pseudomonas* species. *Canadian Journal of Microbiology* **31**: 403-410.

Gross, D.C. (1991) Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. *Annual Review of Phytopathology* **29**: 247-278.

He, P., Shan, L., and Sheen, J. (2007) Elicitation and suppression of microbe-associated molecular pattern-triggered immunity in plant-microbe interactions. *Cell Microbiol* **9**: 1385-1396.

Henke, J.M., and Bassler, B.L. (2004) Three parallel quorum-sensing systems regulate gene expression in *Vibrio harveyi*. *J Bacteriol* **186**: 6902-6914.

Hoang, H.H., Becker, A., and Gonzalez, J.E. (2004) The LuxR homolog ExpR, in combination with the Sin quorum sensing system, plays a central role in *Sinorhizobium meliloti* gene expression. *J Bacteriol* **186**: 5460-5472.

Holden, M.T., Ram Chhabra, S., de Nys, R., Stead, P., Bainton, N.J., Hill, P.J., Manefield, M., Kumar, N., Labatte, M., England, D., Rice, S., Givskov, M., Salmond, G.P., Stewart, G.S., Bycroft, B.W., Kjelleberg, S., and Williams, P. (1999) Quorum-sensing cross talk: isolation and chemical characterization of cyclic dipeptides from *Pseudomonas aeruginosa* and other gram-negative bacteria. *Mol Microbiol* **33**: 1254-1266.

Hwang, I., Li, P.L., Zhang, L., Piper, K.R., Cook, D.M., Tate, M.E., and Farrand, S.K. (1994) TraI, a LuxI homologue, is responsible for production of conjugation factor, the Ti plasmid *N*-acylhomoserine lactone autoinducer. *Proc Natl Acad Sci U S A* **91**: 4639-4643.

Ingle, R.A., Carstens, M., and Denby, K.J. (2006) PAMP recognition and the plant-pathogen arms race. *Bioessays* **28**: 880-889.

Jones, J.D., and Dangl, J.L. (2006) The plant immune system. *Nature* **444**: 323-329.

Kim, J., Kim, J.G., Kang, Y., Jang, J.Y., Jog, G.J., Lim, J.Y., Kim, S., Suga, H., Nagamatsu, T., and Hwang, I. (2004) Quorum sensing and the LysR-type transcriptional activator ToxR regulate toxoflavin biosynthesis and transport in *Burkholderia glumae*. *Mol Microbiol* **54**: 921-934.

Kim, M.G., Kim, S.Y., Kim, W.Y., Mackey, D., and Lee, S.Y. (2008) Responses of *Arabidopsis thaliana* to challenge by *Pseudomonas syringae*. *Mol Cells* **25**: 323-331.

Loh, J., Carlson, R.W., York, W.S., and Stacey, G. (2002) Bradyoxetin, a unique chemical signal involved in symbiotic gene regulation. *Proc Natl Acad Sci U S A* **99**: 14446-14451.

Lugtenberg, B.J., Chin, A.W.T.F., and Bloemberg, G.V. (2002) Microbe-plant interactions: principles and mechanisms. *Antonie Van Leeuwenhoek* **81**: 373-383.

Manefield, M., de Nys, R., Kumar, N., Read, R., Givskov, M., Steinberg, P., and Kjelleberg, S. (1999) Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. *Microbiology* **145 (Pt 2)**: 283-291.

Mathesius, U., Mulders, S., Gao, M., Teplitski, M., Caetano-Anolles, G., Rolfe, B.G., and Bauer, W.D. (2003) Extensive and specific responses of a eukaryote to bacterial quorum-sensing signals. *Proc Natl Acad Sci U S A* **100**: 1444-1449.

McCullen, C.A., and Binns, A.N. (2006) *Agrobacterium tumefaciens* and plant cell interactions and activities required for interkingdom macromolecular transfer. *Annu Rev Cell Dev Biol* **22**: 101-127.

McGowan, S., Sebaihia, M., Jones, S., Yu, B., Bainton, N., Chan, P.F., Bycroft, B., Stewart, G.S., Williams, P., and Salmond, G.P. (1995) Carbapenem antibiotic production in *Erwinia*

carotovora is regulated by CarR, a homologue of the LuxR transcriptional activator. *Microbiology* **141 (Pt 3)**: 541-550.

Mew, T.W., Mew, I.C., and Huang, J. (1984) Scanning electron microscopy of virulent and avirulent strains of *Xanthomonas campestris* pv. *oryzae* on rice leaves. *Phytopathology* **74**: 635-641.

Mew, T.W. (1987) Current status and future prospects of research on bacterial blight of rice. *Annual Review of Phytopathology* **25**: 359-382.

Michael, B., Smith, J.N., Swift, S., Heffron, F., and Ahmer, B.M. (2001) SdiA of Salmonella enterica is a LuxR homolog that detects mixed microbial communities. *J Bacteriol* **183**: 5733-5742.

Miller, M.B., and Bassler, B.L. (2001) Quorum sensing in bacteria. *Annu Rev Microbiol* **55**: 165-199.

Miyajima, K., Tanii, A., and Akita, T. (1983) *Pseudomonas fuscovaginae* sp. nvm. rev. *Int. J. Syst. Bacteriol.* **33**: 656-657.

Nasser, W., Bouillant, M.L., Salmond, G., and Reverchon, S. (1998) Characterization of the *Erwinia chrysanthemi* expI-expR locus directing the synthesis of two *N*-acyl-homoserine lactone signal molecules. *Mol Microbiol* **29**: 1391-1405.

Nealson, K.H., and Markovitz, A. (1970) Mutant analysis and enzyme subunit complementation in bacterial bioluminescence in *Photobacterium fischeri*. *J Bacteriol* **104**: 300-312.

Nealson, K.H., Platt, T., and Hastings, J.W. (1970) Cellular control of the synthesis and activity of the bacterial luminescent system. *J Bacteriol* **104**: 313-322.

Pearson, J.P., Passador, L., Iglewski, B.H., and Greenberg, E.P. (1995) A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **92**: 1490-1494.

Pearson, J.P., Van Delden, C., and Iglewski, B.H. (1999) Active efflux and diffusion are involved in transport of *Pseudomonas aeruginosa* cell-to-cell signals. *J Bacteriol* **181**: 1203-1210.

Pesci, E.C., Milbank, J.B., Pearson, J.P., McKnight, S., Kende, A.S., Greenberg, E.P., and Iglewski, B.H. (1999) Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **96**: 11229-11234.

Pirhonen, M., Flego, D., Heikinheimo, R., and Palva, E.T. (1993) A small diffusible signal molecule is responsible for the global control of virulence and exoenzyme production in the plant pathogen *Erwinia carotovora*. *Embo J* **12**: 2467-2476.

Poplawsky, A.R., and Chun, W. (1997) pigB determines a diffusible factor needed for extracellular polysaccharide slime and xanthomonadin production in *Xanthomonas campestris* pv. *campestris*. *J Bacteriol* **179**: 439-444.

Rahme, L.G., Ausubel, F.M., Cao, H., Drenkard, E., Goumnerov, B.C., Lau, G.W., Mahajan-Miklos, S., Plotnikova, J., Tan, M.W., Tsongalis, J., Walendziewicz, C.L., and Tompkins, R.G. (2000) Plants and animals share functionally common bacterial virulence factors. *Proc Natl Acad Sci U S A* **97**: 8815-8821.

Reverchon, S., Bouillant, M.L., Salmond, G., and Nasser, W. (1998) Integration of the quorum-sensing system in the regulatory networks controlling virulence factor synthesis in *Erwinia chrysanthemi*. *Mol Microbiol* **29**: 1407-1418.

Rott, P., and Notteghem, J.L. (1989) Identification and Characterization of *Pseudomonas fuscovaginae*, the Causal Agent of Bacterial Sheath Brown Rot, from Madagascar and Other Countries. *Plant Disease* **73**: 133-137.

Rott, P. (1991) Identification of *Pseudomonas fuscovaginae* with biochemical, serological, and pathogenicity tests. *Plant Disease* **75**: 843-846.

Schaefer, A.L., Val, D.L., Hanzelka, B.L., Cronan, J.E., Jr., and Greenberg, E.P. (1996) Generation of cell-to-cell signals in quorum sensing: acyl homoserine lactone synthase activity of a purified *Vibrio fischeri* LuxI protein. *Proc Natl Acad Sci U S A* **93**: 9505-9509.

Shaw, P.D., Ping, G., Daly, S.L., Cha, C., Cronan, J.E., Jr., Rinehart, K.L., and Farrand, S.K. (1997) Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc Natl Acad Sci U S A* **94**: 6036-6041.

Shiu, S.H., and Bleecker, A.B. (2001) Receptor-like kinases from *Arabidopsis* form a monophyletic gene family related to animal receptor kinases. *Proc Natl Acad Sci U S A* **98**: 10763-10768.

Shiu, S.H., and Bleecker, A.B. (2003) Expansion of the receptor-like kinase/Pelle gene family and receptor-like proteins in *Arabidopsis*. *Plant Physiol* **132**: 530-543.

Slock, J., VanRiet, D., Kolibachuk, D., and Greenberg, E.P. (1990) Critical regions of the *Vibrio fischeri* luxR protein defined by mutational analysis. *J Bacteriol* **172**: 3974-3979.

Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.X., Zhu, L.H., Fauquet, C., and Ronald, P. (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. *Science* **270**: 1804-1806.

Soto, M.J., Sanjuan, J., and Olivares, J. (2006) Rhizobia and plant-pathogenic bacteria: common infection weapons. *Microbiology* **152**: 3167-3174.

Steindler, L., and Venturi, V. (2007) Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol Lett* **266**: 1-9.

Sweeney, M., and McCouch, S. (2007) The complex history of the domestication of rice. *Ann Bot (Lond)* **100**: 951-957.

Swings, J., Van den Mooter, M., Vauterin, L., Hoste, B., Gillis, M., Mew, T.W., and Kersters, K. (1990) Reclassification of the causal agents of bacterial blight (*Xanthomonas campestris pv. oryzae*) and bacterial leaf streak (*Xanthomonas campestris pv. oryzicola*) of rice as pathovars of Xanthomonas oryzae (ex Ishiyama 1922) sp. nov., nom. rev. *Int. J. Syst. Bacteriol.* **40**: 309-311.

Tanii, A., Miyajima, K., and Akita, T. (1976) The sheath brown rot disease of rice and its causal bacterium *Pseudomonas fuscovaginae* sp. nov. *Ann. Phytopathol. Soc. Jpn.* **42**: 540-548.

Teplitski, M., Robinson, J.B., and Bauer, W.D. (2000) Plants secrete substances that mimic bacterial *N*-acyl homoserine lactone signal activities and affect population density-dependent behaviors in associated bacteria. *Mol Plant Microbe Interact* **13**: 637-648.

Teplitski, M., Chen, H., Rajamani, S., Gao, M., Merighi, M., Sayre, R.T., Robinson, J.B., Rolfe, B.G., and Bauer, W.D. (2004) *Chlamydomonas reinhardtii* secretes compounds that mimic bacterial signals and interfere with quorum sensing regulation in bacteria. *Plant Physiol* **134**: 137-146.

van der Geer, P., Hunter, T., and Lindberg, R.A. (1994) Receptor protein-tyrosine kinases and their signal transduction pathways. *Annu Rev Cell Biol* **10**: 251–337.

Vojnov, A.A., Slater, H., Newman, M.A., Daniels, M.J., and Dow, J.M. (2001) Regulation of the synthesis of cyclic glucan in *Xanthomonas campestris* by a diffusible signal molecule. *Arch Microbiol* **176**: 415-420.

Weber, E., Ojanen-Reuhs, T., Huguet, E., Hause, G., Romantschuk, M., Korhonen, T.K., Bonas, U., and Koebnik, R. (2005) The type III-dependent Hrp pilus is required for productive interaction of *Xanthomonas campestris* pv. *vesicatoria* with pepper host plants. *J Bacteriol* **187**: 2458-2468.

Xavier, K.B., and Bassler, B.L. (2003) LuxS quorum sensing: more than just a numbers game. *Curr Opin Microbiol* **6**: 191-197.

Zeigler, R.S., and Alvarez, E. (1987) Bacterial sheath brown rot of rice by *Pseudomonas fuscovaginae* in Latin America. *Plant Disease* **71**: 592-597.

Zipfel, C., and Felix, G. (2005) Plants and animals: a different taste for microbes? *Curr Opin Plant Biol* **8**: 353-360.

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

2.1 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.

2.2 INTRODUCTION

Bacteria which belong to the genus *Xanthomonas* are Gram-negative γ-Proteobacteria and are significant pathogens for 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 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 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).

In the genus *Xanthomonas* to our knowledge there are no reports of the presence of AHL QS systems. On the other hand, QS has been reported in *Xanthomonas campestris* pv. *campestris* and *Xoo* to occur via a signalling molecule designated DSF

(diffusible signal factor) (Chatterjee and Sonti, 2002; Dow *et al.*, 2003; He *et al.*, 2006). DSF has been characterized as *cis*-11-methyl-2-dodecenoic acid (Wang *et al.*, 2004) 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 this study we investigated whether *Xoo* produces and responds to AHLs and present evidence (i) that a set of *Xoo* isolates do not produce AHLs, (ii) of the existence of a conserved LuxR QS regulator in *Xoo* which we designated OryR, (iii) that OryR is important for rice virulence (iv) that OryR is induced by macerated rice and (v) that OryR likely interacts with a plant signal.

2.3 RESULTS AND DISCUSSION

2.3.1 Rice pathogenicity tests on *Xanthomonas oryzae* pv. *oryzae* isolates

The pathogenicity of the 23 *Xoo* isolates from India was tested on the susceptible rice line IR24 and 3 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 1 of the 23 tested isolates, while IRBB5 and IRBB21 were resistant to 7 and 10 *Xoo* isolates, respectively (Table 1).

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 relevant sites of India. However, the adult resistance earlier demonstrated in 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).

Table 1: *Xanthomonas oryzae* pv. *oryzae* strains used. Bacteria strains was provided by M. Höfte laboratory. Pathogenicity tests on four different rice varieties was performed by J. Bigirimana.

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	N/A
BX043	Unknown	Unknown	Unknown	Unknown	S	N/A	N/A	N/A	(Goel, 2002)
KACC10331	Korea§	Unknown	Unknown	Unknown	S	N/A	N/A	N/A	(Lee 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).

2.3.2 *Xanthomonas oryzae* pv. *oryzae* rice pathogenic bacteria does not produce AHLs

Using bacterial biosensor AHL detector strains described in the Experimental Procedures section, all the *Xanthomonas oryzae pv. oryzae* (*Xoo*) isolates listed in Table 1 were used initially to test by growth in solid media in plate streak assay for AHL production (Hwang *et al.*, 1994).

The bacterial biosensor *C. violaceum* CVO26 induces the production of violacein when certain AHL signal molecules are present, *E. coli* (pSB401) and *E. coli* (pSB1075) induce bioluminescence, *A. tumefaciens* NT1 (pZLR4) and *P. fluorescens* 1855(pSF105)(pSF107) induce β-galactosidase production. These four AHL biosensor strains ensure that a wide range of AHLs can be 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 of which the genome 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 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 likely does not produce AHL molecules. It cannot be excluded that *Xoo* could be producing

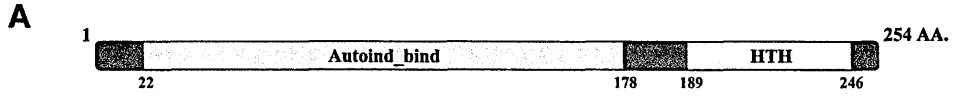
AHLs at extremely low amounts which cannot be detected or the AHL biosensors used here do not respond to the AHL molecules potentially produced by *Xoo*.

2.3.3 The genome of *Xanthomonas oryzae* pv. *oryzae* contains a potential quorum sensing orphan LuxR-family member

The genome sequences of two *Xoo* strains, designated as KACC10331 and MAFF311018, have been published (Lee *et al.*, 2005; Ochiai *et al.*, 2005) and very recently a third strain, PX099A, has also been sequenced (Salzberg *et al.*, 2008). 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, Vibrio fischeri* and *Vibrio 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 of which the genome sequence 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 characteristics signatures of an AHL-dependent response regulator. OryR is 254 amino acids long and it contains an autoinducer binding domain (Pfam03472) from position 22-178 and HTH domain from 189-246 with the conserved region of LuxR family regulators (Figure 1).

Interestingly, although its function is unknown, an ORF highly similar (over 90%) to OryR is also present in closely related *Xanthomonas campestris* pv. *vesicatoria* (Q3BQU7), *Xanthomonas axonopodis* pv. *citri* (Q8P1BO) and *Xanthomonas campestris* pv. *campestris* (Q4UX59). All *oryR* genes have been annotated as single transcriptional units and not part of operons.



B

```
Consensus                *->deealkdllerfakrlGfdyyaYgvlppkpptrpqeqlkpliisNYP
                              + ++d+ +   + l +dy    +       +p     +++  N P
OryR-Autoind_bind   22    LDRVFRDVCALGFQSLVYDYAPVPLSMEGALITP----TVFMQRNAP   64

Consensus                deWverYlannYfaiDPvvkhalssisPfsWs--------ddlfdaslsl
                         ++  +   +++Y  +DPv  +a ++ +Pf+Ws         ++ + +
OryR-Autoind_bind   65 GDMQHVWCEHGYYQHDPVQQRATRRNTPFVWSyrtdgdcaGVEYVGG---  111

Consensus                esrrvfeeArefGlrnGlTfPlhganGnlgmLSfarseedlsdferrils
                         + r+v +   ++G+++G+T+Plh + G+++ +S a ++ + +++ r ++s
OryR-Autoind_bind  122 QHRQVTRYLCDSGMGTGVTVPLHLPGGAFATFSAAIDAVA-AEALRLAES  160

Consensus                lklalqmllilaherlsr<-*
                         ++l + +l++++++r+
OryR-Autoind_bind  161 QLLPFLLLAHAFQARAQE   178
```

C

```
Consensus                *->ldsLspREreVLrllaqGksNkeIAdeLgiSekTVkvHrsnimrKLn
                              +L++Rere+L++ a+G+++k IA++L++S  TV+ H+ ++ rKL+
OryR-HTH           189    HIPLTRRERECLQYSAKGLTSKRIAAALNRSTATVNLHLNSAARKLG  235

Consensus                vhsrvelirla<-*
                         +++rve+++
OryR-HTH           236 ARNRVEAVVRG   246
```

Figure 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. Position 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). (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 acid with similar properties (http://www.sanger.ac.uk/Software/Pfam/).

OryR displays approximately 50% similarity to several LuxR family proteins of various *Pseudomonas syringae* plant pathogenic species (Q48E34; QOEE63; Q87WK7 and Q4ZNM6) and to several LuxR proteins belonging to members of the *Rhizobium* genus (Q92M411 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 LuxI 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 being SdiA of *E. coli* and *Salmonella enterica* which enables these bacteria which do not synthesize AHLs, to nevertheless 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).

2.3.4 oryR is conserved in *Xanthomonas 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 *Nco*I 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 (Figure 2). Of the 7 which did not give a signal, we analysed by Western analysis whether they contained an OryR-like protein; 5 of the 7 did respond to anti-OryR antibody having a protein band of the expected size (Figure 1 - Appendix). It was concluded that *oryR* is widely but not completely conserved within the *Xoo* species.

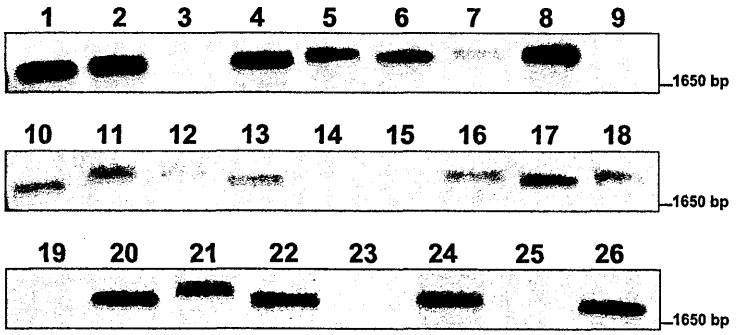


Figure 2: High stringency Southern analysis using *oryR* DNA as a probe against NcoI digested chromosomal DNA from 26 *Xoo* strains. The number correspond to the following *Xoo* strains (see Table 1 for further details): 1, XAPT.43; 2, XAPC.5; 3, XAPC.10; 4, XAPC.11; 5, XAPC.12; 6, XAPC.13; 7, XAPC.14; 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.

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

The *oryR* gene was mutated in three *Xoo* strains in order to understand its role in rice pathogenicity. The three *Xoo* strains were KACC10331 of which the genome 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.

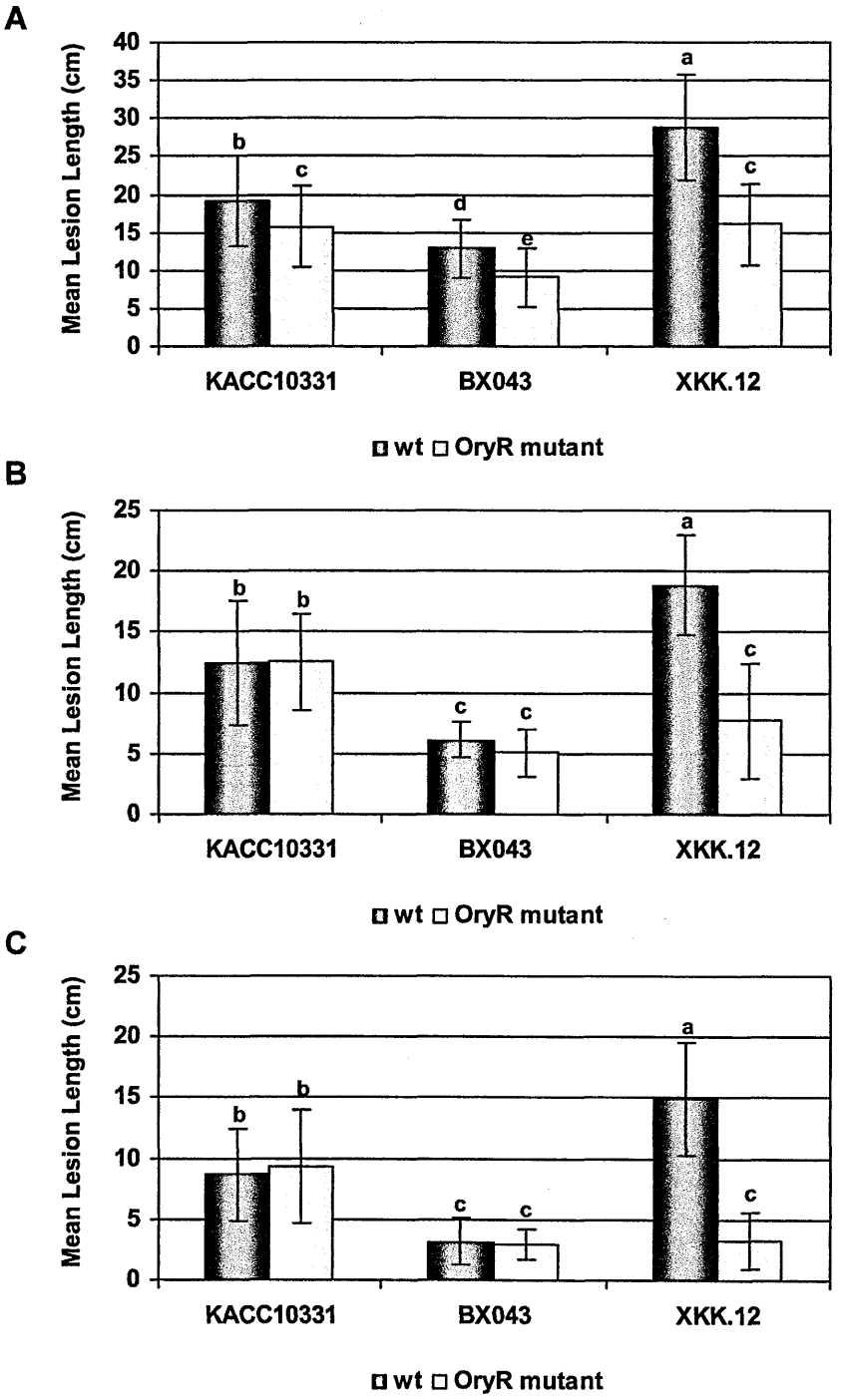


Figure 3: *oryR* mutations affect *Xoo* virulence. Presentations of the results of leaf lesion lengths caused by three *Xoo* and *oryR* mutant derivatives. The results are expressed as means ± STDEV; different letters are significantly different using the Mann-Whitney comparison test performed on lesion length data, see text for details (statistical analysis was performed by J. Bigirimana). (A) A 10^9 CFU/mL *Xoo* inoculum was used, (B) 10^8 CFU/mL, (C) 10^7 CFU/mL. See text for details.

The assays were performed on rice leaves at very high concentration of inoculum (10^9 cfu/ml) as well as in lower concentration (10^8 and 10^7 cfu/ml). As depicted in Figure 3, the XKK.12 *oryR* mutants were less virulent when compared to 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 lower virulence only at high inoculum since under these conditions the wild type strains displayed strong virulence.

2.3.6 OryR is solubilized by macerated rice

The study of LuxR family quorum sensing 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 against 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 overexpressing and purifying OryR (see experimental procedures), 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 solubilize. 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 (i.e. C_4-, C_6-, C_8-, C_{10}-, and C_{12}-AHL), several 3oxo-AHLs (i.e. C_6-3oxo-, C_8-3oxo-, C_{10}-

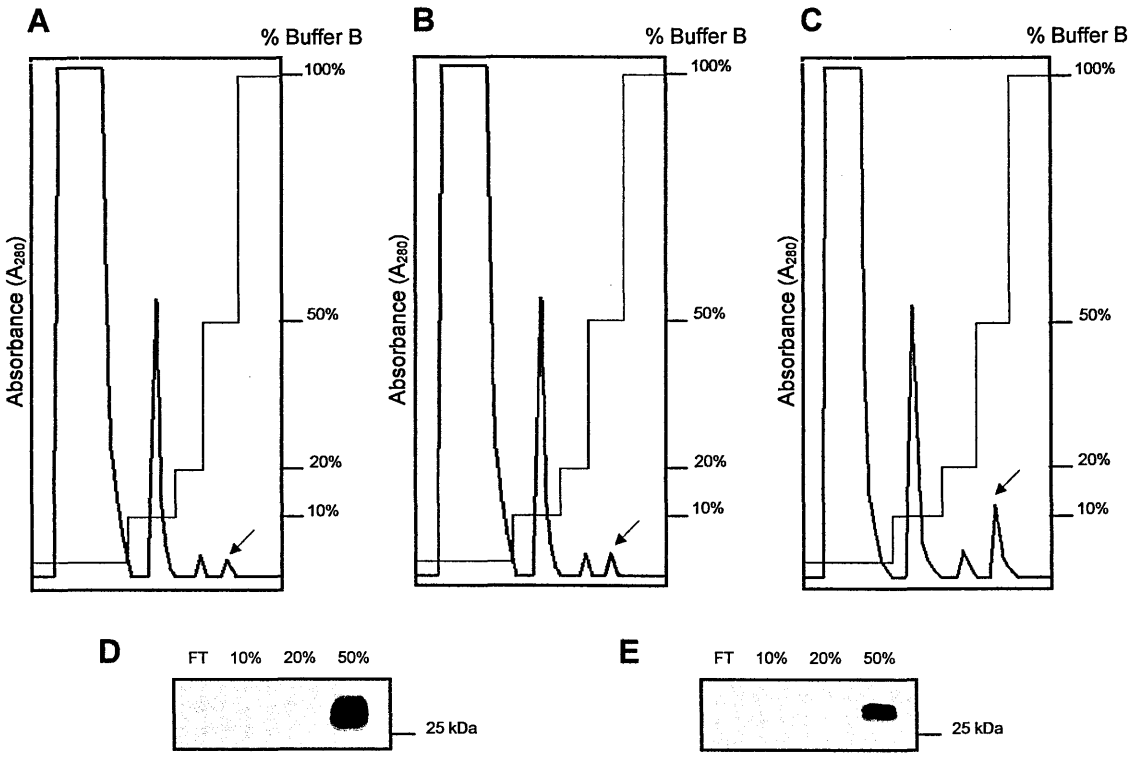


Figure 4: Solubility studies of His6-OryR. (A) Affinity chromatography using LB as growth medium for *E. coli* harbouring pPQEORYR and pREP-4. Arrow indicates putative low amounts of OryR protein eluted at 50% of Buffer B, corresponding to 135 mM of imidazole (see Experimental Procedures section) or 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 section). (D) Western blot analysis of each elution peak from His6-OryR affinity chromatography (Figure 4-C) using anti-OryR antibody. (E) The same result was obtained using anti 6x-His monoclonal antibody. FT: flow through.

3oxo-, and C_{12}-3oxo-AHLs) and several 3OH-AHLs molecules (i.e. C_6-3OH-, C_8-3OH-, C_{10}-3OH-, and C_{12}-3OH-AHL). No OryR protein solubilization was observed when the protein was over-expressed in the presence of these molecules (data not shown).

Though 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 resulted rice powder was added to LB medium (see experimental procedures). After nickel affinity chromatography, pure His-tagged OryR in native form was eluted at 135mM of imidazole (Figure 4C). The presence of OryR in the elution peak was confirmed by Western blot analysis using both, anti-OryR antibody (Figure 4D) and anti 6x-His antibody (Figure 4E). As control, no OryR solubilization peak was observed in the presence of LB medium alone (Figure 4A) or LB medium supplemented tobacco (*Xoo* non-host plant) macerated powder (Figure 4B). This result raises the hypothesis that a 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).

2.3.7 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 M9-casamino acids medium (see experimental procedures), no OryR protein was detected with Western analysis (Figure 5A), whereas when grown in M9-casamino acids medium supplemented with macerated rice, OryR levels increased significantly and could be clearly detected (Figure 5A). These results indicate that *oryR* is probably induced *in planta* thus most likely affecting target gene expression when *Xoo* is in rice. OryR was not detected when *Xoo* KACC10331 was grown in M9-casamino acids medium in the presence of a cocktail

of exogenously provided AHLs (Figure 5A) indicating that they do not affect the expression of *oryR*. It cannot be excluded however that some component(s) of 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 versus the profile of the *oryR* mutant KACC10331ORYR to determine if any secreted proteins were regulated by OryR.

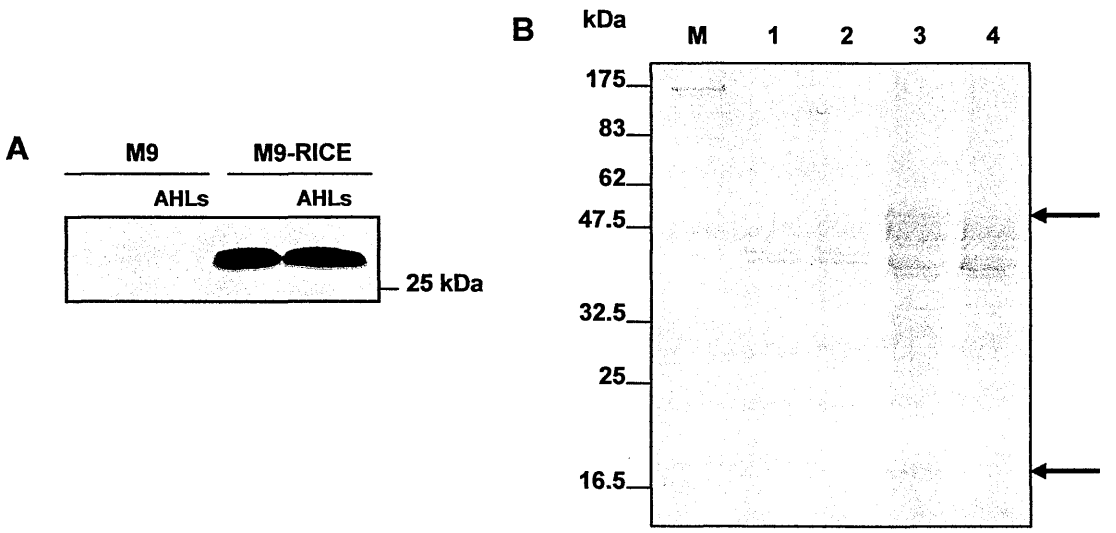


Figure 5: (A) OryR levels in *Xoo* grown in different conditions. Similar amounts of stationary phase *Xoo* cells grown in (1) M9-casamino acids medium alone or with a cocktail of AHLs (C4-, C6-, C8-, C10- C12-AHLs, same for 3-oxo-AHLs and for 3-OH-AHLs all added at 1 µM) and (2) M9-casamino acids medium in the presence of macerated rice and 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 M9-casamino acids medium (lane 1), *Xoo* KACC10331ORYR grown in M9-casamino acids medium (lane 2), *Xoo* KACC10331 grown in minimal M9-casamino acids medium supplemented with macerated rice (lane 3) and *Xoo* KACC10331ORYR grown in M9-casamino acids medium supplemented macerated rice (lane 4). The arrows indicated 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.

As depicted in Figure 5B, in KACC10331ORYR two secreted proteins of approximately 60 kDa and 20 kDa were present at lower levels when compared to 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 spectrometry 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).

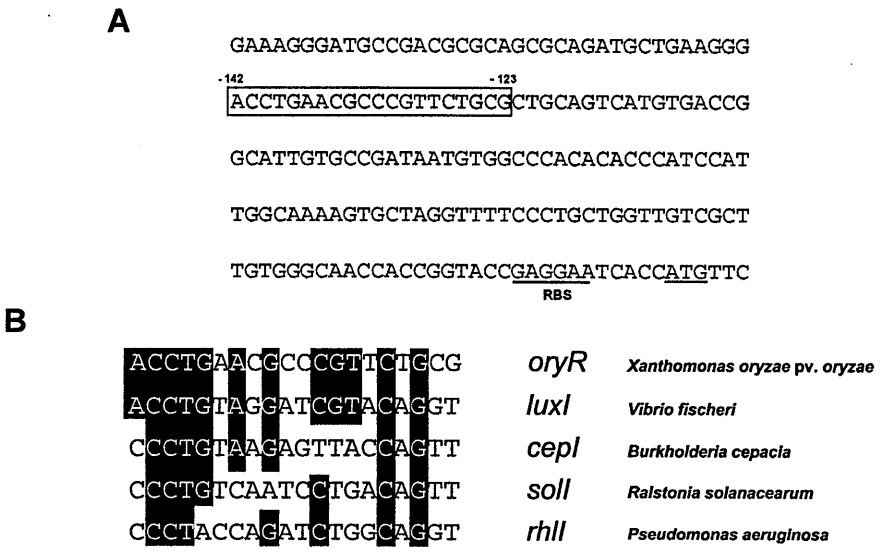


Figure 6: Putative *lux* box in the *oryR* promoter region. (A) Nucleotide sequence of the promoter region of the *oryR* gene; underlined is the RBS (putative Shine Delgarno sequence) and the ATG of the translational start codon. Boxed is the putative *lux* box and the numbers indicate the distance relative 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.

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).

2.4 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 quorum sensing 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 produce 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 since 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 quorum sensing 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 on the possibility that OryR responds to AHL compounds produced by other bacteria or by AHL mimic compounds derived from rice (Degrassi *et al.*, 2007). While attempts to solubilize recombinant OryR using several different AHL compounds failed, OryR solubilization was achieved in the presence of rice extract. This suggested 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 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 further *Xoo* targets of OryR and the molecule(s) to which it responds.

2.5 EXPERIMENTAL PROCEDURES

2.5.1 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 liter; 8 g peptone, 2 g yeast extract, 2 g K_2HPO_4, 0.5 g KH_2PO_4, 0.25 g $MgSO_4.7H_20$, 0.5% glucose w/v), PS medium (Tsuchiya *et al.*, 1982) or in M9 minimal medium with the addition of casamino acids 0,3% w/v (Sambrook *et al.*, 1989) and, if necessary, macerated rice plants 2,5% w/v (cv. Baldo). Forty five day-old rice plants were frozen at –80 and macerated with pestle and mortar, rice powder was then added to the liquid medium

and filtered after sterilization. Kanamycin 100 μg/ml was used for *Xoo* mutants 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.

2.5.2 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 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).

2.5.3 Purification, detection and visualization of signal (AHL) molecules

The purification, detection and visualisation of AHLs signal molecules from culture supernatants were performed essentially as previously described [(Steindler and Venturi, 2007) and references therein]. Synthetic C_4-AHL to C_{12}-AHL were purchased from Fluka Chemie AG (Buchs, CH) and C_6-3oxo-AHL to C_{12}-3oxo-AHL and C_6-3OH-AHL to C_{12}-3OH-AHLs were purchased from the laboratory of Prof. Paul Williams (University of Nottingham, UK).

2.5.4 Inactivation of *oryR* of *Xanthomonas 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 a *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 blot analysis (Figure 2 - Appendix).

2.5.5 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, D). 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, Hilden, D) yielding pQEORYR.

Proteins were transferred onto 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, USA) and after incubation with the second HRP-labelled antibody the proteins were detected with the 3-3'-diaminobenzidine (DAB) tetrahydrochloride (Sigma, St. Louis, Missouri, USA). 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 8,000*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 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 sulfate-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 analyzed by mass spectrometry (an internal sequence analysis of the protein spots was performed by using an electronspray ionization mass spectrometer LCQ DECA XP, ThermoFinnigam), proteins were then identified by analysis of the peptides and by using the *Xoo* protein data banks.

2.5.6 OryR over-expression and purification

For OryR overexpression, a single 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 pre-warmed medium. The expression was induced by adding isopropyl-β-D-thiogalactoside 1 mM at OD_{600} 0.6 and was carried on for 3 hours 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 instructions of the supplier (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 [10mM (Buffer A) and 250 mM (Buffer B)]. Protein concentration was determined by using a Bradford assay (BioRad).

2.5.7 Bacterial leaf blight virulence assays on rice plants

Xanthomonas oryzae pv. *oryzae* isolates were grown on Sucrose Peptone Agar medium 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 demineralised water. Pathogenicity tests on the *Xoo* strains in Table 1 were carried out on rice cultivar IR24 and 3 NILs (Near-Isogenic Lines) IRBB5, IRBB7 and IRBB21 (from International Rice Research Institute IRRI). 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 light-dark

photoperiod. Plants were weekly fertilized with 5 g $(NH_4)_2SO_4$ and 10 g $FeSO_4.7H_2O$ per 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 4 leaves were used per plant and 1 leaf per rice line was clipped using sterile H_2O for the control. Five to 6 plants were inoculated per isolate and were kept for 18 hours in humid chambers (≥ 92% R.H.) 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 lesions. Plants were divided into 3 classes: resistant with lesion length of 0 to 3 cm, intermediate, with 3 to 9 cm and susceptible with more than 9 cm. Rice cultivar IR24 was used to assess the virulence of *Xoo* strains KACC10331, BXO43, XKK.12 and their respective *oryR* mutants. Twenty to twenty-five leaves were infected by each of the 6 strains. Experiments were performed in triplicate. Lesion length data did not fulfil the requirements for ANOVA and were statistically analysed with the SPSS program using the non-parametric tests: Kruskal-Wallis multiple comparison completed by the Mann-Whitney test.

2.6 ACKNOWLEDGEMENTS

S.F. is supported by an ICGEB fellowship. V.V.'s laboratory in the ICGEB Biosafety Outstation in Ca'Tron and J.B. ICGEB fellowship is also supported by the Fondazione Cassamarca, Treviso, Italy.

2.7 REFERENCES

Ahmer, B.M. (2004) Cell-to-cell signalling in *Escherichia coli* and *Salmonella enterica*. *Mol Microbiol* **52**: 933-945.

Alexeyev, M.F. (1999) The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. *Biotechniques* **26**: 824-826, 828.

Barber, C.E., Tang, J.L., Feng, J.X., Pan, M.Q., Wilson, T.J., Slater, H., Dow, J.M., Williams, P., and Daniels, M.J. (1997) A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol Microbiol* **24**: 555-566.

Better, M., Lewis, B., Corbin, D., Ditta, G., and Helinski, D.R. (1983) Structural relationships among *Rhizobium meliloti* symbiotic promoters. *Cell* **35**: 479-485.

Birnboim, H.C. (1983) A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol* **100**: 243-255.

Camara, M., Williams, P., and Hardman, A. (2002) Controlling infection by tuning in and turning down the volume of bacterial small-talk. *Lancet Infect Dis* **2**: 667-676.

Camilli, A., and Bassler, B.L. (2006) Bacterial small-molecule signaling pathways. *Science* **311**: 1113-1116.

Chai, Y., and Winans, S.C. (2004) Site-directed mutagenesis of a LuxR-type quorum-sensing transcription factor: alteration of autoinducer specificity. *Mol Microbiol* **51**: 765-776.

Chatterjee, S., and Sonti, R.V. (2002) *rpfF* mutants of *Xanthomonas oryzae* pv. *oryzae* are deficient for virulence and growth under low iron conditions. *Mol Plant Microbe Interact* **15**: 463-471.

Collins, C.H., Arnold, F.H., and Leadbetter, J.R. (2005) Directed evolution of *Vibrio fischeri* LuxR for increased sensitivity to a broad spectrum of acyl-homoserine lactones. *Mol Microbiol* **55**: 712-723.

Degrassi, G., Devescovi, G., Solis, R., Steindler, L., and Venturi, V. (2007) *Oryza sativa* rice plants contain molecules that activate different quorum-sensing *N*-acyl homoserine lactone biosensors and are sensitive to the specific AiiA lactonase. *FEMS Microbiol Lett* **269**: 213-220.

Dow, J.M., Crossman, L., Findlay, K., He, Y.Q., Feng, J.X., and Tang, J.L. (2003) Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. *Proc Natl Acad Sci U S A* **100**: 10995-11000.

Fuqua, C., and Greenberg, E.P. (2002) Listening in on bacteria: acyl-homoserine lactone signalling. *Nat Rev Mol Cell Biol* **3**: 685-695.

Fuqua, C. (2006) The QscR quorum-sensing regulon of *Pseudomonas aeruginosa*: an orphan claims its identity. *J Bacteriol* **188**: 3169-3171.

Fuqua, W.C., Winans, S.C., and Greenberg, E.P. (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176**: 269-275.

He, Y.W., Wang, C., Zhou, L., Song, H., Dow, J.M., and Zhang, L.H. (2006) Dual signaling functions of the hybrid sensor kinase RpfC of *Xanthomonas campestris* involve either phosphorelay or receiver domain-protein interaction. *J Biol Chem* **281**: 33414-33421.

Hoang, H.H., Becker, A., and Gonzalez, J.E. (2004) The LuxR homolog ExpR, in combination with the Sin quorum sensing system, plays a central role in *Sinorhizobium meliloti* gene expression. *J Bacteriol* **186**: 5460-5472.

Hwang, I., Li, P.L., Zhang, L., Piper, K.R., Cook, D.M., Tate, M.E., and Farrand, S.K. (1994) TraI, a LuxI homologue, is responsible for production of conjugation factor, the Ti plasmid *N*-acylhomoserine lactone autoinducer. *Proc Natl Acad Sci U S A* **91**: 4639-4643.

Jha, G., Rajeshwari, R., and Sonti, R.V. (2007) Functional interplay between two *Xanthomonas oryzae* pv. *oryzae* secretion systems in modulating virulence on rice. *Mol Plant Microbe Interact* **20**: 31-40.

Kauffman, H., Reddy, A.P.K., Hsieh, S.P.Y., and Merca, S.D. (1973) An improved technique for evaluating resistance of rice varieties to *Xanthomonas oryzae*. *Plant Dis. Rep.* **57**: 537-541.

Leach, J.E., and White, F.F. (1996) Bacterial avirulence genes. *Annu Rev Phytopathol* **34**: 153-179.

Leach, J.E., Vera Cruz, C.M., Bai, J., and Leung, H. (2001) Pathogen fitness penalty as a predictor of durability of disease resistance genes. *Annu Rev Phytopathol* **39**: 187-224.

Lee, B.M., Park, Y.J., Park, D.S., Kang, H.W., Kim, J.G., Song, E.S., Park, I.C., Yoon, U.H., Hahn, J.H., Koo, B.S., Lee, G.B., Kim, H., Park, H.S., Yoon, K.O., Kim, J.H., Jung, C.H., Koh, N.H., Seo, J.S., and Go, S.J. (2005) The genome sequence of *Xanthomonas oryzae* pathovar *oryzae* KACC10331, the bacterial blight pathogen of rice. *Nucleic Acids Res* **33**: 577-586.

Lequette, Y., Lee, J.H., Ledgham, F., Lazdunski, A., and Greenberg, E.P. (2006) A distinct QscR regulon in the *Pseudomonas aeruginosa* quorum-sensing circuit. *J Bacteriol* **188**: 3365-3370.

Milton, D.L., Chalker, V.J., Kirke, D., Hardman, A., Camara, M., and Williams, P. (2001) The LuxM homologue VanM from *Vibrio anguillarum* directs the synthesis of *N*-(3-hydroxyhexanoyl)homoserine lactone and *N*-hexanoylhomoserine lactone. *J Bacteriol* **183**: 3537-3547.

Ochiai, H., Inoue, Y., Takeya, M., Sasaki, A., and Kaku, H. (2005) Genome sequence of *Xanthomonas oryzae* pv. *oryzae* suggests contribution of large numbers of effector genes and insertion sequences to its race diversity. *Jpn Agric. Res. Q* **39**: 275-287.

Qi, Z., and Mew, T.W. (1985) Adult-plant resistance of rice cultivars to bacterial blight. *Plant Dis.* **69**: 896-898.

Salzberg, S.L., Sommer, D.D., Schatz, M.C., Phillippy, A.M., Rabinowicz, P.D., Tsuge, S., Furutani, A., Ochiai, H., Delcher, A.L., Kelley, D., Madupu, R., Puiu, D., Radune, D., Shumway, M., Trapnell, C., Aparna, G., Jha, G., Pandey, A., Patil, P.B., Ishihara, H., Meyer, D.F., Szurek, B., Verdier, V., Koebnik, R., Dow, J.M., Ryan, R.P., Hirata, H., Tsuyumu, S., Won Lee, S.,

Ronald, P.C., Sonti, R.V., Van Sluys, M.A., Leach, J.E., White, F.F., and Bogdanove, A.J. (2008) Genome sequence and rapid evolution of the rice pathogen *Xanthomonas oryzae* pv. *oryzae* PXO99A. *BMC Genomics* **9**: 204.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*

Schuster, M., Urbanowski, M.L., and Greenberg, E.P. (2004) Promoter specificity in *Pseudomonas aeruginosa* quorum sensing revealed by DNA binding of purified LasR. *Proc Natl Acad Sci U S A* **101**: 15833-15839.

Shen, Y., and Ronald, P. (2002) Molecular determinants of disease and resistance in interactions of *Xanthomonas oryzae* pv. *oryzae* and rice. *Microbes Infect* **4**: 1361-1367.

Shiner, E.K., Rumbaugh, K.P., and Williams, S.C. (2005) Inter-kingdom signaling: deciphering the language of acyl homoserine lactones. *FEMS Microbiol Rev* **29**: 935-947.

Sidhu, G.S., and Khush, G.S. (1978) Dominance reversal of a bacterial blight resistance gene in some rice cultivars. *Phytopathology* **68**: 461–463.

Steindler, L., and Venturi, V. (2007) Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol Lett* **266**: 1-9.

Swings, J., Van den Mooter, M., Vauterin, L., Hoste, B., Gillis, M., Mew, T.W., and Kersters, K. (1990) Reclassification of the causal agents of bacterial blight (*Xanthomonas campestris pv. oryzae*) and bacterial leaf streak (*Xanthomonas campestris pv. oryzicola*) of rice as pathovars of *Xanthomonas oryzae* (ex Ishiyama 1922) sp. nov., nom. rev. *Int. J. Syst. Bacteriol.* **40**: 309-311.

Tsuchiya, K., Mew, T.W., and Wakimoto, S. (1982) Bacteriological and. pathological characteristics of wild-types and induced mutants of *Xanthomonas campestris* pv. *oryzae*. *Phytopathology* **72**: 43-46.

Urbanowski, M.L., Lostroh, C.P., and Greenberg, E.P. (2004) Reversible acyl-homoserine lactone binding to purified *Vibrio fischeri* LuxR protein. *J Bacteriol* **186**: 631-637.

Vandamme, P., Pot, B., Gillis, M., de Vos, P., Kersters, K., and Swings, J. (1996) Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol Rev* **60**: 407-438.

Walters, M., and Sperandio, V. (2006) Quorum sensing in *Escherichia coli* and *Salmonella*. *Int J Med Microbiol* **296**: 125-131.

Wang, L.H., He, Y., Gao, Y., Wu, J.E., Dong, Y.H., He, C., Wang, S.X., Weng, L.X., Xu, J.L., Tay, L., Fang, R.X., and Zhang, L.H. (2004) A bacterial cell-cell communication signal with cross-kingdom structural analogues. *Mol Microbiol* **51**: 903-912.

Waters, C.M., and Bassler, B.L. (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* **21**: 319-346.

Zhu, J., and Winans, S.C. (2001) The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization. *Proc Natl Acad Sci U S A* **98**: 1507-1512.

OryR is a LuxR-family protein involved in inter-kingdom signalling between pathogenic *Xanthomonas oryzae* pv. *oryzae* and rice

3.1 SUMMARY

Xanthomonas oryzae pv. *oryzae* (*Xoo*), the causal agent of bacterial leaf blight (BLB) in rice, contains a regulator in the genome, called OryR, which belongs to the *N*-acyl homoserine lactone (AHL) dependent quorum sensing (QS) sub-family of LuxR-homolog proteins. We previously reported that *Xoo* however does not make AHLs, does not possess a LuxI-family AHL synthase and that the OryR protein is solubilized by a compound present in rice. In this study we provide further evidence that OryR interacts with a rice signal molecule (RSM) and that the concentration of RSM increases when rice is infected with *Xoo*. We also report three OryR target promoters which are regulated differently; (i) the neighbouring proline iminopeptidase (*pip*) virulence gene which is positively regulated by OryR in the presence of the RSM, (ii) the *oryR* promoter which is negatively autoregulated independently of the RSM and (iii) the 1,4-β-cellobiosidase *cbsA* gene which is positively regulated by OryR independently of the RSM. It is also shown that the RSM for OryR is small in size, not related to AHLs and not able to activate the broad range AHL-biosensor *A. tumefaciens* NT1(pZLQR). Furthermore OryR does not regulate the production of the quorum sensing diffusible signal factor (DSF) present in the *Xanthomonas* genus. OryR has therefore unique features being an important regulator involved in the inter-kingdom communication between host and pathogen.

3.2 INTRODUCTION

The species *Xanthomonas oryzae* includes two pathovars, *oryzae* and *oryzicola* which are pathogens of rice, are closely related and were initially named as pathovars of *Xanthomonas campestris* (Swings et al., 1990). *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) are Gram-negative rod-shape bacteria causing bacterial leaf blight (BLB), one of the most important diseases of rice. BLB is a vascular disease where *Xoo* grows and colonizes the xylem vessels eventually clogging them; several virulence associated determinants have been found including exopolysaccharide production, hypersensitive response and pathogenicity (*hrp*) genes (Cho *et al.*, 2007; Lee *et al.*, 2008; Shen and Ronald, 2002).

Many Gram-negative bacteria possess a form of gene regulation involving cell-cell communication, also known as quorum sensing (QS), via *N*-acyl homoserine lactones (AHLs) signalling molecules. A typical AHL QS system is most commonly mediated by two proteins belonging to the LuxI-LuxR protein families; LuxI-type proteins are AHL synthases and LuxR-family proteins are modular sensor-response regulators. In an AHL QS system, AHLs interact directly at high bacterial cell density, i.e. 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 (Fuqua *et al.*, 2001). AHL QS has been studied in many bacterial species and shown to provide a significant advantage to a community of bacteria by adapting to environmental conditions, enhancing its defense capabilities against other microorganisms or eukaryotic resistance mechanisms (Camara *et al.*, 2002; Waters and Bassler, 2005).

Xoo does not produce AHL QS signalling molecules; however we recently reported that it possesses a protein, called OryR, which is related to the LuxR-family of AHL QS regulators (Ferluga *et al.*, 2007). In fact OryR is a modular protein having an *N*-acyl homoserine lactone (AHL) domain and a helix-turn-helix domain both typical of the LuxR-family subgroup of quorum sensing (QS) regulators (Ferluga *et al.*, 2007; Fuqua *et al.*, 2001). OryR does not have a typical cognate AHL LuxI family synthase present in the genome and can therefore be regarded as an unpaired or orphan LuxR-type response regulator (Fuqua, 2006; Walters and Sperandio, 2006). OryR was shown not to bind the most common AHLs, however it appears to bind a compound present in the rice plant. This was concluded following the observation that the OryR protein was not solubilized by many of the structurally different AHLs but OryR solubilization was achieved in the presence of rice extract (Ferluga *et al.*, 2007). It was also determined that OryR plays a role in *Xoo* rice virulence since an *oryR* mutant was less able to cause the BLB symptoms (Ferluga *et al.*, 2007). A highly similar protein to OryR, designated as XccR, has also been reported in the plant pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) which has been associated with *Xcc* pathogenicity and regulates *in planta* the neighbouring proline iminopeptidase (*pip*) virulence gene (Zhang *et al.*, 2007). Studies on the *xccR/pip* locus revealed that XccR associates with a plant factor and functions as a transcriptional activator binding to the *lux* box present in the promoter of the *pip* gene.

Plants have been reported to produce compounds that are able to act as agonists or antagonist to bacterial AHL QS systems and hence have been called AHL mimics

(Bauer and Mathesius, 2004). Halogenated furanones from the marine red alga *Delisea pulchra*, structurally similar to the C_4-AHL molecule, were able to competitively bind the LuxR homologue proteins having inhibitory functions (Givskov *et al.*, 1996; Manefield *et al.*, 1999). Additionally AHL mimics from the unicellular green alga *Chlamydomonas reinhardtii* and several other plants, including rice, were able to stimulate gene expression via LuxR-family AHL sensors/regulators (Degrassi *et al.*, 2007; Teplitski *et al.*, 2004). To date the structure of these plant compounds is unknown and it cannot be excluded that similar molecules are involved in inter-kingdom signalling with OryR of *Xoo* and rice.

In this study we provide further evidence of the presence of a molecule in rice which interacts with OryR and that the presence of this molecule increases when rice is infected with *Xoo*. We also provide evidence of three OryR target genes and how they are regulated in response to the presence of macerated rice. It was also established that the OryR regulatory network does not affect DSF production, the signal molecule found and characterized in *Xanthomonas campestris* pv. *campestris* and present in multiple *Xanthomonas* species. OryR therefore has unique features, being an important player in plant-bacteria interaction through the detection and response of a small diffusible plant compound.

3.3 RESULTS

3.3.1 The presence of xylem sap, collected from *Xoo* infected rice, in the growth media increases OryR protein solubility

The OryR primary structure contains domains typical of quorum sensing LuxR-family regulators: an AHL-binding domain in its *N*-terminus and an HTH-DNA-binding motif at the C-terminus. It was previously shown that when LuxR-family QS proteins are over-expressed they are highly insoluble, while in the presence of, and bound to, the cognate AHL molecule they become soluble (Schuster *et al.*, 2004; Urbanowski *et al.*, 2004; Vannini *et al.*, 2002). OryR, like other LuxR-family regulators, was found to be highly insoluble, but it became soluble when expressed in *E. coli* grown in the presence of macerated rice (Ferluga *et al.*, 2007). It was therefore postulated that an unknown rice signal molecule (RSM) was present in rice and was able to interact with OryR solubilizing the protein. Many structurally different AHLs were unable to solubilize OryR indicating that most likely OryR did not bind AHLs (Ferluga *et al.*, 2007).

To verify the presence, and possibly the concentration, of the RSM in infected rice, an OryR solubilization assay was performed using rice previously infected with *Xoo*. As *Xoo* is a pathogen colonizing and infecting the xylem it is most likely that the RSM is present in this plant environment. Rather than using total macerated rice as used previously (Ferluga *et al.*, 2007), we now harvested the xylem sap from *Xoo* XKK.12 infected rice plants, three, six, ten and fourteen days after infection and from non-infected rice plants as control, as described in Materials and Method section. *E. coli* M15-pQEORYR, over-expressing His_6-OryR (Ferluga *et al.*, 2007), was then grown

in the presence of xylem sap isolated from these four time intervals and it was established whether a soluble form of the OryR was present via Western blot analysis using an anti His-tag antibody. Highest amounts of soluble OryR were found when bacteria were grown in the presence of xylem sap collected 10 days after *Xoo* infection. This result indicated that in the xylem either maximum concentrations of RSM and/or OryR levels were reached approximately ten days after *Xoo* infection (Figure 1).

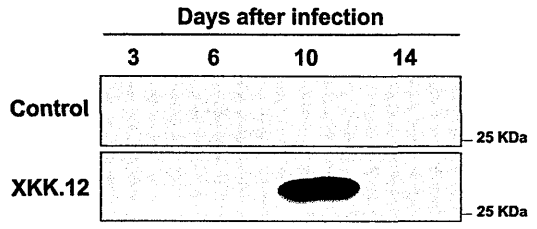


Figure 1: Western blot analysis of soluble His-tag OryR expressed in *E. coli* grown in media containing infected xylem sap recovered from rice at various time points (ie. 3, 6, 10 or 14 days). A soluble form of OryR was detectable most when expressed in *E. coli* in the presence of xylem sap recovered from rice 10 days after infection (see text for details).

3.3.2 Gene promoter studies of the *oryR/pip* region: OryR regulates *pip* in response to a rice signal molecule (RSM)

Proline iminopeptidase (PIP) production is regulated by XccR, the homolog of OryR, in *Xanthomonas campestris* pv. *campestris* (*Xcc*) (Zhang *et al.*, 2007). The biological function of PIP is currently unclear; this enzyme can catalyze the removal of the *N*-terminal proline from small peptides or proteins and is widely distributed among bacteria. PIP in *Xcc* has been shown to be a virulence factor as *pip* mutants were less pathogenic to cabbage since they were less fit to spread and grow in the vascular

system (Zhang *et al.*, 2007). The *pip* gene in both *Xcc* and *Xoo* is genetically linked to the *xccR* and *oryR* gene respectively (Figure 2).

Interestingly, both *pip* promoters of *Xoo* and *Xcc* contain well conserved *lux*-boxes typically found in AHL QS regulated target genes in Gram-negative bacteria. The putative *lux* box sequence in *Xoo pip* promoter is centred at position –71 from the start codon (Figure 2-A), and was found to be highly similar to the experimentally determined *lux* box in the *Xcc pip* promoter (Figure 2-B) (Zhang *et al.*, 2007).

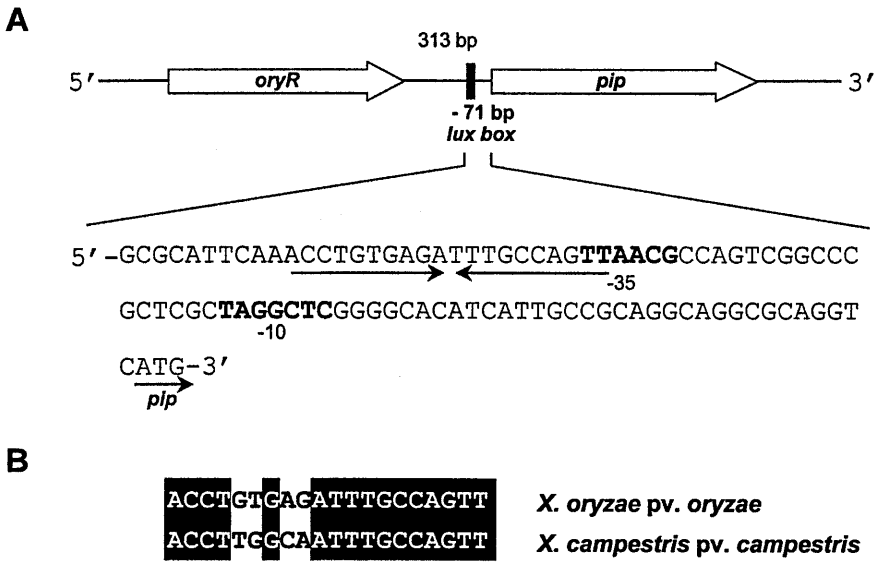


Figure 2: (A) Analysis of the *pip* promoter locus in *Xoo*. The 313 bp intergenic region upstream from the *pip* gene contains a putative palindromic *lux* box sequence centred at –71 bp from the starting codon as indicated by the arrows. The hypothetical –35 and –10 regions are highlighted in bold. (B) Alignment of the two putative *pip lux*-boxes identified in *Xoo* and *Xcc*.

To verify whether OryR is able to regulate the *pip* promoter in *Xoo*, an IncW *gusA* promoter probe plasmid designated pSS122, stable in *Xanthomonas*, has been constructed here as described in the Materials and Methods section. The *pip* promoter was then cloned upstream of the promoterless β-glucuronidase reporter gene in

pSS122 generating pPIP122. *Xoo* strain XKK.12 and *oryR* mutant derivative XKK.12ORYR carrying pPIP122 were grown under different conditions in the presence and absence of macerated rice. Our previous studies determined that no OryR protein was detected when *Xoo* was grown in a minimal M9 medium, however the protein was highly expressed when adding macerated rice to the minimal medium demonstrating that *oryR* expression was most likey induced *in planta* (Ferluga *et al.*, 2007). We observed however the presence of the OryR protein when *Xoo* was grown in the rich PYS medium indicating that some component(s) in this complex medium was probably in part inducing *oryR* expression. We therefore performed *pip* promoter activity studies in PYS rich medium with and without the addition of macerated rice thus ensuring that OryR is always present with the only difference being the presence of macerated rice.

It was determined that *pip* promoter activity in the wild type strain was approximately five times higher when macerated rice was present in the medium, highlighting that most likely a compound present in rice was pivotal for *pip* transcription. Significantly, no promoter activity in any of the conditions tested was detected in the *Xoo oryR* mutant indicating that the compound, or rice signal molecule (RSM), present in macerated rice was necessary to activate *pip* promoter via OryR (Figure 3).

Having established that 10-day xylem sap from *Xoo* infected rice resulted in the highest amount of OryR solubilization (see above), we determined whether *pip* promoter activity further increased in the presence of infected rice in the growth medium. *Xoo* XKK.12 (pPIP122) cells were therefore grown in medium containing macerated 10-day-old *Xoo* infected rice and β-glucuronidase assays were then

performed. As shown in Figure 3, the activity of the *pip* promoter in the presence of macerated, infected rice was approximately ten times higher when compared to the activity of the control and a further 2-fold increase when macerated un-infected rice was used; this suggest that 10-day old infected rice probably contained larger amounts of RSM produced and recognized by OryR. No β-glucuronidase expression was observed in the *oryR* mutant *Xoo* XKK.12ORY (pPIP122) further confirming that the *pip* gene was tightly regulated by OryR (Figure 3).

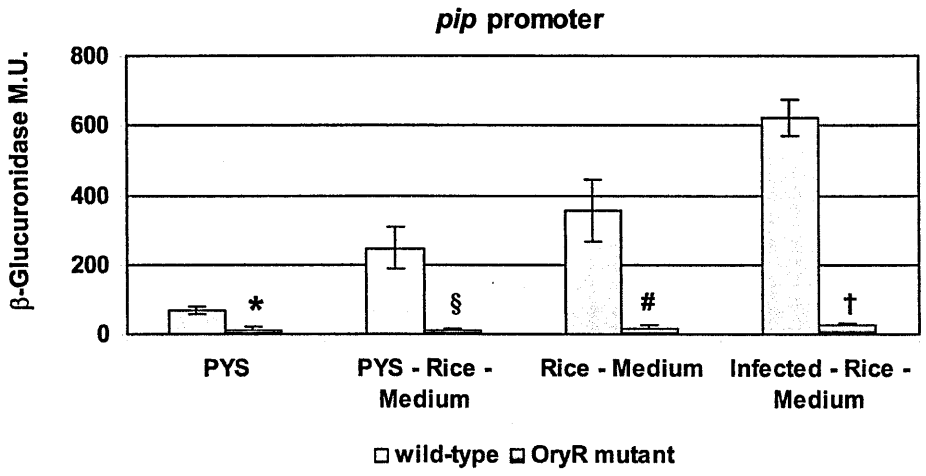


Figure 3: *pip* gene promoter activity measured in *Xoo* XKK.12 harbouring the reporter plasmid pPIP122 and grown in different media, with and without the presence of macerated rice. 'PYS' refers to a rich medium (see text for details); 'PYS-Rice-Medium' refers to PYS in the presence of macerated rice; 'Rice-Medium' refers to macerated rice in distilled sterile water; 'Infected-Rice-Medium' refers to rice which was infected with *Xoo* for 10 days prior to maceration. The highest *pip* promoter activity was measured when *Xoo* XKK.12(pPIP122) was grown in the presence of infected rice. No promoter activity was detected in the *oryR* mutant *Xoo* XKK.12ORY (pPIP122). The results are expressed as means ± STDEV, n=3. *, p< 0,002; §, p< 0,003; #, p< 0,003; †, p< 4 x 10^{-5}, compared to the parental strain.

To further verify whether the *pip* promoter was also functional *in planta*, *Xoo* XKK.12 (pPIP122) was used for rice infection and bacterial cells were then recovered from rice plants one week after infection and β-glucuronidase assays were performed. Although most of the bacterial cells recovered from the infected plants 10-days after

inoculation had lost the promoter-probe plasmid, significant β-glucuronidase activity was detected, clearly indicating a high level of *pip* promoter expression *in planta* (Figure 3 - Appendix).

3.3.3 OryR negatively regulates its own expression in a rice independent manner

In order to determine whether OryR was able to regulate its own activity, β-glucuronidase assay was performed in PYS rich medium on *Xoo* XKK.12 and on *oryR*

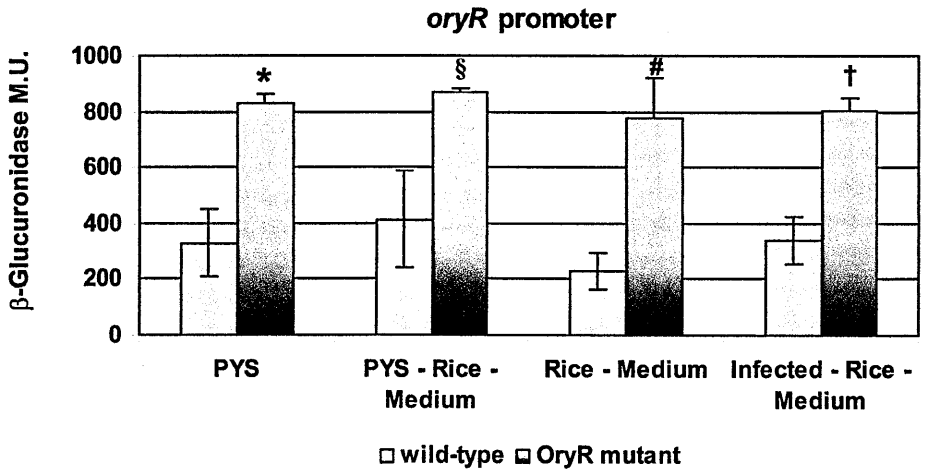


Figure 4: *oryR* promoter activity measured in *Xoo* XKK.12(pORY122) and in the *oryR* mutant *Xoo* XKK.12ORY (pORY122) in presence and in the absence of macerated rice in the growth medium. 'PYS' refers to a rich medium (see text for details); 'PYS-Rice-Medium' refers to PYS in the presence of macerated rice; 'Rice-Medium' refers to macerated rice in distilled sterile water; 'Infected-Rice-Medium' refers to rice which was infected with *Xoo* for 10 days prior to maceration. The results are expressed as means ± STDEV, n=3. *, p< 0,002; §, p< 0,012; #, p< 0,002; †, p< 0,001, compared to the parental strain.

mutant derivative XKK.12ORY containing the pORY122 plasmid which is a transcriptional fusion of the *oryR* promoter with the promoterless *uidA* encoding the

β-glucuronidase gene. In the *Xoo oryR* mutant, the expression from the *oryR* promoter was two-fold higher when compared to levels obtained in the wild type *Xoo* strain, showing that OryR was acting as a negative autoregulator. The promoter activity profile was similar with or without the presence of macerated rice in the medium (Figure 4), indicating that OryR was able to negatively regulate its own expression and thus act as a transcriptional regulator also in the absence of the RSM.

3.3.4 1,4-β-cellobiosidase expression is OryR-dependent

1,4-β-cellobiosidase (CbsA) catalyses the hydrolysis of 1,4-β-D-glucosidic linkages in cellulose, releasing cellobiose from the non-reducing ends of the chains. This hydrolytic enzyme was identified as one of the *Xoo* secreted proteins involved in virulence as the ability to cause lesions in rice by *Xoo cbs* mutants was reduced (Jha *et al.*, 2007). Our previous studies showed that maximal production of the secreted CbsA from *Xoo* KACC10331 wild-type strain occurred when macerated rice was present in the culture medium in the presence of a functional *oryR* gene (Ferluga *et al.*, 2007).

To verify whether OryR was regulating the expression of *cbsA*, we performed the β-glucuronidase assays on *Xoo* XKK.12 and on XKK.12ORY containing the pCBS122 plasmid, where the *cbs* promoter was cloned upstream of the promoterless *uidA* gene. Interestingly, the *cbs* promoter activity in wild type *Xoo* XKK.12 in rich PYS medium was approximately 50% reduced when macerated rice was added to the medium, however no β-glucuronidase expression was observed in the *oryR* mutant *Xoo* XKK.12ORY (pPCBS122)(Figure 5).

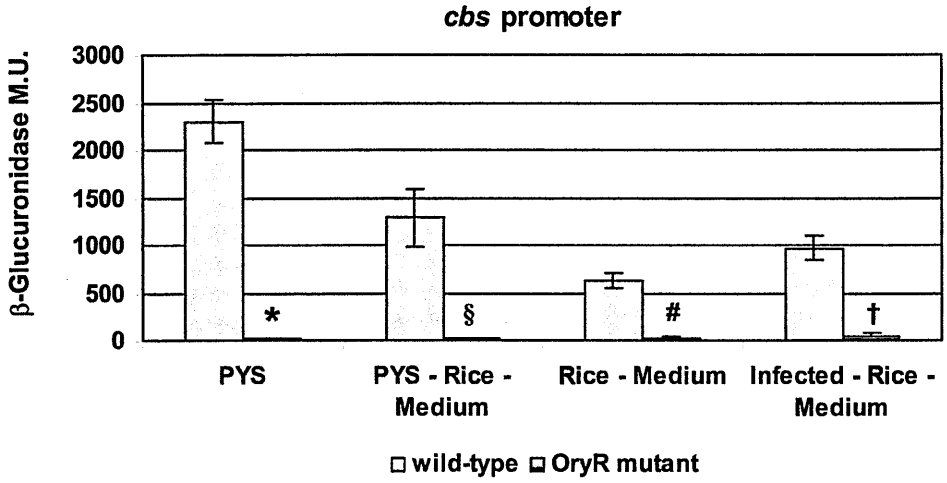


Figure 5: *cbs* promoter activity measured in *Xoo* XKK.12(pCBS122) in different growth media. 'PYS' refers to a rich medium (see text for details); 'PYS-Rice-Medium' refers to PYS in the presence of macerated rice; 'Rice-Medium' refers to macerated rice in distilled sterile water; 'Infected-Rice-Medium' refers to rice which was infected with *Xoo* for 10 days prior to maceration. The results are expressed as means ± STDEV, n=3. *, $p < 6 \times 10^{-5}$; §, p< 0,002; #, $p < 2 \times 10^{-4}$; †, $p < 3 \times 10^{-4}$, compared to the parental strain.

This result indicated that OryR regulated *cbs* expression and hence 1,4-beta-cellobiosidase (CbsA) production independent of the RSM molecule. The reduction of *cbs* promoter activity in the presence of macerated rice was surprising since the Cbs protein is most abundant when *Xoo* is grown in the presence of macerated rice (Ferluga *et al.*, 2007). It cannot therefore be excluded that *cbs*/Cbs expression undergoes post-transcriptional regulation.

3.3.5 OryR does not regulate production of DSF: the quorum sensing signal molecule produced by *Xoo*

QS has been reported in *Xanthomonas campestris* pv. *campestris* to occur via a signaling molecule designated DSF (diffusible signal molecule) (Barber *et al.*, 1997; Slater *et al.*, 2000). DSF has been characterized as *cis*-11-methyl-2-dodecenoic acid which is synthesized by the *rpfF* gene; DSF signaling is involved in the regulation of

biofilm dispersal and production of virulence factors (Barber *et al.*, 1997; Dow *et al.*, 2003). A two-component regulatory system designated RpfC/RpfG is involved in the perception and transduction of the DSF signal to target genes (He *et al.*, 2006). Since *Xoo* also contains the *rpf* cluster in the genome and produces DSF (Chatterjee and Sonti, 2002), it was of interest to determine if OryR was involved and interconnected with DSF production in *Xoo*. Using a previously described DSF sensor strain (Barber *et al.*, 1997), we established DSF production in *Xoo* XKK.12 and *Xoo* XKK.12ORY testing protease and endoglucanase activity as described in the Materials and Methods section. DSF levels were comparable in the wild-type and *oryR* mutant derivative demonstrating that OryR was not involved in the regulation of QS via DSF production (Figure 4 - Appendix). To further confirm that OryR was not involved in DSF production, the *rpfF* promoter controlling the DSF biosynthesis gene was cloned in pSS122 upstream of the promoterless *uidA*, generating pRPFF122. β-glucuronidase assays were then performed on XKK.12(pRPFF122) and *Xoo* XKK.12ORY (pRPFF122) cells. No differences in activity were observed in all growth conditions tested further confirming that OryR does not regulate DSF production (data not shown).

We were interested to also determine whether DSF production in *Xoo* was influenced by the presence of macerated rice in the growth media. Extraction of DSF from *Xoo* XKK.12 and *Xoo* XKK.12ORY grown in PYS rich medium, in macerated rice medium and in infected macerated rice medium established that there were no differences in DSF production in XKK.12 compared to *Xoo* XKK.12ORY in all

growth conditions tested (Figure 4 - Appendix), meaning that probably DSF production does not alter *in planta*.

3.3.6 RSM is a small molecule probably unrelated to AHLs

Previous studies of OryR solubility have shown that the RSM which was able to bind OryR was probably not an *N*-acyl-homoserine lactone type molecule (AHL) (Ferluga *et al.*, 2007). This evidence was based on the fact that structurally different AHLs could not solubilize OryR. To further confirm this data we analysed the OryR target *pip* promoter activity when adding structurally different AHLs (C_4-, C_6-, C_8-, C_{10}-, C_{12}-, C_6 ,3oxo-, C_8-3oxo-, C_{10}-3oxo-, C_{12}-3oxo-, C_6-3OH-, C_8-3OH-, C_{10}-3OH- and C_{12}-3OH-AHL) to the culture medium to a final concentration of 2 μM each in independent experiments.

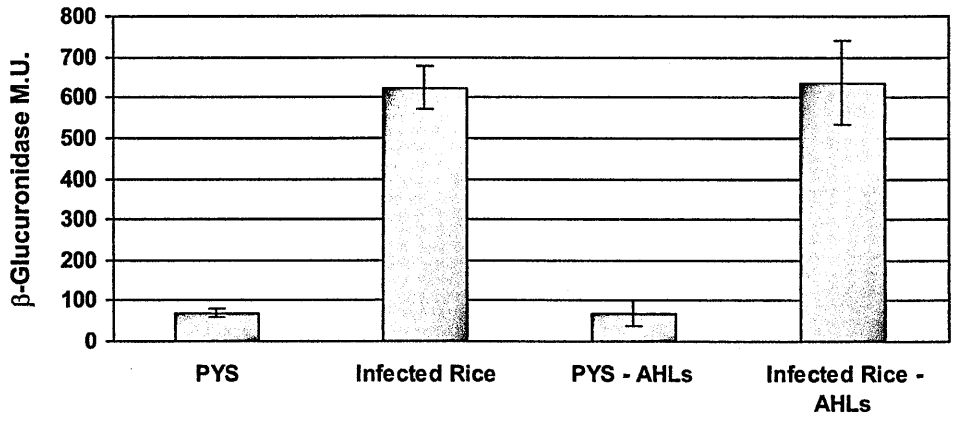


Figure 6: β-Glucuronidase *pip* promoter activity measured in *Xoo* XKK.12 (pPIP122) in the presence of a mixture of structurally different AHLs. 'PYS' refers to a rich medium (see text for details); 'Infected Rice' refers to rice which was infected with *Xoo* for 10 days prior to maceration; 'PYS-AHLs' refer to a rich medium containing 2 μM each of the 15 most structurally common AHLs; 'Infected Rice-AHLs' refers to rice which was infected with *Xoo* for 10 days prior to maceration containing 2 μM each of the 15 most structurally common AHLs. No *pip* promoter activation and no binding competition was observed in the presence of AHLs. The results are expressed as means ± STDEV, n=3. Statistical analysis, performed comparing "PYS" values with "PYS-AHLs" values and comparing "Infected rice" values with "Infected Rice-AHLs" values, resulted not to be statistically significant (p≥ 0,05).

No *pip* gene promoter induction was observed and no competition for the OryR binding site, since the β-glucuronidase production of *Xoo* XKK.12 (pPIP122) cells by RSM was not reduced in the presence of any of the structurally different AHLs (Figure 6).

To verify that the RSM was a small molecule, media containing the RSM were fractionated according to molecular size by progressive filtrations (see Materials and Methods).

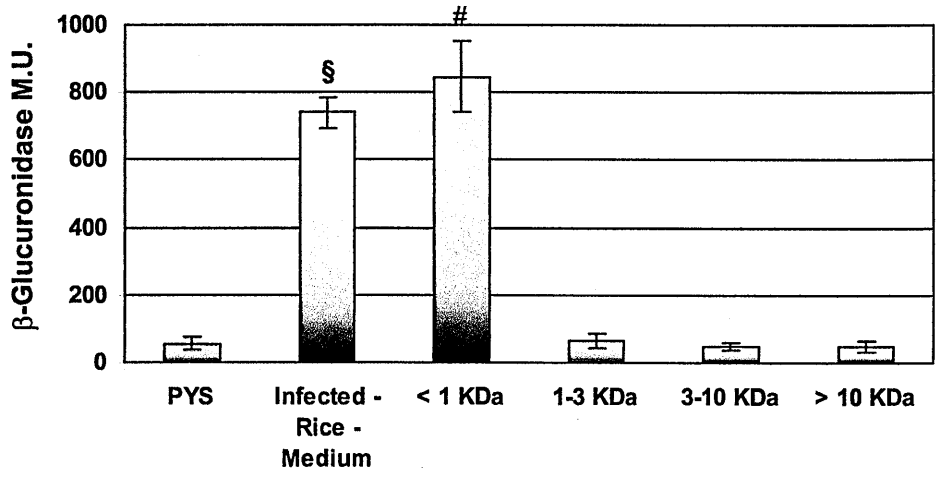


Figure 7: *pip* promoter activity measured in *Xoo* XKK.12(pPIP122) in rich medium, in the presence of macerated rice or in different fractions of filtered infected macerated rice medium. 'PYS' refers to *Xoo* (pPIP122) grown in rich medium (see text for details); 'Infected-Rice-Medium' refers to *Xoo* (pPIP122) grown in rice which was infected with *Xoo* for 10 days prior to maceration; < 1 kDa refers to *Xoo* (pPIP122) grown in the presence of a filtrate from macerated rice excluding all molecules larger than 1kDa; 1-3 kDa refers to *Xoo* (pPIP122) grown in the presence of a filtrate form macerated rice including molecules within this size range; 3-10 kDa 1-3 kDa refers to *Xoo* (pPIP122) grown in the presence of a filtrate from macerated rice including molecules within this size range; >10 kDa refers to *Xoo* (pPIP122) grown in the presence of a filtrate from macerated rice including molecules larger than 10kDa. Promoter activity through β-Glucuronidase activity was only detected in the medium containing the <1KDa fraction indicating that RSM is a molecule smaller than 1KDa. The results are expressed as means ± STDEV, n=3. §, $p < 1,5 \times 10^{-5}$ compared to "PYS" value; #, $p < 2 \times 10^{-4}$ compared to "PYS" value. Statistical analysis of "1-3 KDa", 3-10 KDa" and " >10 KDa" values, compared to "PYS" value, resulted not to be statistically significant (p≥ 0,05).

The β-glucuronidase assay of *Xoo* XKK.12 (pPIP122) was then performed on the four fractions obtained and it was clearly established that strong *pip* promoter activation occurred only in the fraction <1 KDa, clearly indicating the RSM was a small molecule (Figure 7).

3.3.7 RSM does not act as an AHL QS mimic

Several studies have reported that plants contain molecules able to activate bacterial AHL QS systems, however their structure is currently unknown (Bauer and Mathesius, 2004). To study whether RSM could act as an AHL mimic, activating a quorum sensing LuxR-family protein, *A. tumefaciens* NT1(pZLQR) was used as an AHL biosensor strain for its ability to recognize a broad range of different AHL molecules (Cha *et al.*, 1998). In the presence of an active AHL molecule, TraR activates the transcription of the β-galactosidase reporter gene present in the pZLQR plasmid. *A. tumefaciens* NT1(pZLQR) was grown in the presence of the <1 KDa fraction containing the active RSM as described above. As a positive control a mix of different AHL molecules was added to the culture medium whereas medium alone was assayed as negative control. As expected β-galactosidase activity was detected in the presence of AHLs while no background activity was found in the medium alone. A very slight increase in β-galactosidase activity was measured in the presence of macerated rice (Figure 8). This increase in activity, even if was not so evident, appears to be statistically significant. So, we cannot exclude that this fraction from the rice plant contained molecules able to weakly activate AHL QS systems.

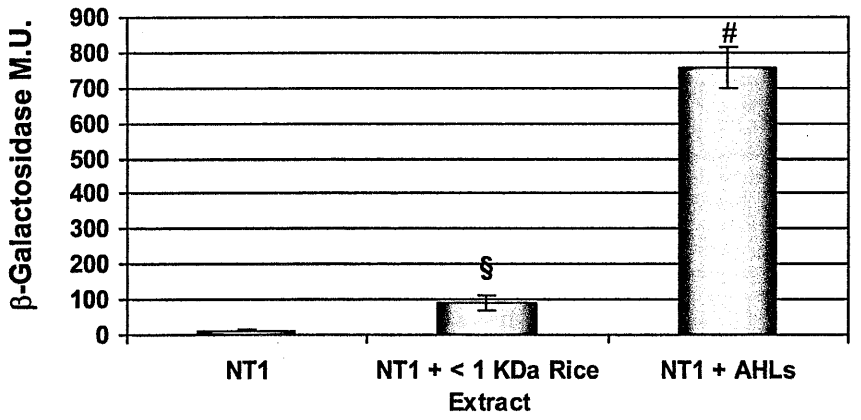


Figure 8: β-Galactosidase activity measured in the *A. tumefaciens* NT1(pZLQR) AHL biosensor grown in the presence of the <1KDa fraction obtained from macerated, infected rice. Growth medium in the presence and absence of AHLs were used as positive and negative controls respectively. The results are expressed as means ± STDEV, n=3. §, p< 0,004 compared to "NT1" value; #, p< 2,5 x 10^{-5} compared to "NT1" value.

3.4 DISCUSSION

In this study we demonstrated that the LuxR-family OryR regulatory protein present in *Xoo* is responding to a small rice signal molecule (RSM). OryR displays the typical modular structure of quorum sensing LuxR-family response-regulator proteins; it contains at the N-terminus an AHL binding domain and a helix-turn-helix DNA binding domain at the C-terminus. The primary structure of OryR however, just like XccR of *Xanthomonas campestris* pv. *campestris*, does not display sequence similarity in the AHL-binding domain in two highly conserved amino acids (Trp57 and Tyr61) which structural analysis in TraR of *Agrobacterium tumefaciens* have shown to be involved in AHL binding (Zhang *et al.*, 2002). Trp57 forms a hydrogen bond with the keto group of AHL whereas Tyr61 is part of the β-sheet surface, (the AHL-binding domain consists of an α/β/α 'sandwich') important for interactions with the fatty acyl chain of the AHL. This lack of conservation in these two important

amino acids might have evolved allowing OryR to bind to a structurally different molecule present in the rice plant and allowing this protein to be involved in inter-kingdom signalling. Very recently Zhang et al. (2007) have carefully investigated the presence of OryR and XccR-like proteins of *Xoo* and *Xcc* respectively in other bacterial species and have importantly determined that related proteins form a distinct group comprising proteins, for example, from *Pseudomonas syringae*, *P. fluorescens* and *Rhizobium leguminosarum*. All the bacterial species possessing an OryR related protein live in close association with plants, thus it is reasonable to postulate that they might be interacting with similar plant-derived signal molecules.

This work has shown that the RSM is present in the collected xylem sap. Experiments have shown that highest levels of OryR solubility were obtained by providing to the growth media xylem sap from 10-day old *Xoo* infected plants. In addition OryR promoter activation of the *pip* target gene was highest when adding macerated *Xoo* infected rice to the growth media, resulting in a 10-fold activation compared to 5-fold when adding un-infected macerated rice. These results indicate that the RSM is most likely present in higher concentrations in rice when it is infected by *Xoo*, possibly via a defence response to the infection. In fact plants synthesize an extremely large set of low molecular weight secondary metabolites in response to pathogen attack (Dixon, 2001) and it is therefore possible that the RSM interacting with OryR is one of these molecules. Since salicylic acid is known to be an important signalling molecule involved in microbial defence, we tested whether 5 μM of salicylic acid added to the growth medium could induce OryR activity of the *pip* gene promoter; no induction was observed (data not shown) hence it was concluded that it does not bind OryR.

Due to the very numerous low molecular weight secondary metabolites produced by plants, some are at very low concentrations, it will be a major challenge identifying the molecule(s) interacting with the OryR subfamily of LuxR family regulators. Probably the RSM is not related to AHLs since competition experiments with AHLs and RSM did not alter the ability of OryR to activate the *pip* promoter, in addition no AHL was able to solubilize OryR.

In this study we report three target promoters of OryR. Firstly, the *pip* gene target is located adjacent to the *oryR* gene and the *pip* promoter contains a very well conserved *lux* box. The *oryR/pip* locus with a *lux* box is very well conserved among plant associated bacteria which possess an *oryR*-like gene (Zhang *et al.*, 2007). The *lux* box in the *pip* promoter of *Xcc* has been shown to be functional and regulated by XccR *in planta* (Zhang *et al.*, 2007). The *pip* promoter in *Xoo* is tightly positively regulated by OryR in response to the RSM and due to the very high conservation with the *xccR/pip* locus of *Xcc* it is most likely that the *lux* box is functional and that OryR once bound to the RSM then binds to the *lux* box and directly activates transcription of the *pip* gene. The PIP enzyme was shown to be a virulence factor in *Xcc*. We did not determine if it was so also for *Xoo*. However, due to the high identity of the two loci and because both *Xoo* and *Xcc* are vascular pathogens, it is probable that PIP in *Xoo* is also associated with virulence. Secondly, OryR negatively regulated its own transcription since the *oryR* promoter displayed a 2-fold increase in activity in the *oryR* mutant; importantly this increase was independent of the presence of rice extract indicating that OryR can probably also influence transcription in the absence of the RSM. It is not known whether this OryR autoregulation is direct or indirect. We could

not detect a clear *lux* box in the *oryR* promoter. However this does not exclude the possibility of OryR direct regulation since *lux* boxes can have several sequence variations. Thirdly, OryR regulated the expression of the 1,4-β-cellobiosidase *cbsA* gene which encodes a secreted hydrolytic enzyme involved in *Xoo* virulence (Jha *et al.*, 2007). Our previous studies showed that, in *Xoo oryR* mutants, there was significantly less CbsA in the extracellular medium (Ferluga *et al.*, 2007). The *cbs* promoter displayed strong promoter activity in rich medium which was dependent on OryR since in the *Xoo oryR* mutant the promoter activity decreased very significantly; the reason for this is currently unknown. The *cbs* promoter activity decreased in the wild type strain by approximately 50% when macerated rice was added to the medium. The *cbs* promoter was therefore positively regulated by OryR but unlike the *pip* promoter, in a rice-independent way; again we cannot exclude that OryR regulates the *cbs* promoter indirectly as we cannot detect a clear *lux* box in its promoter region. The three OryR promoter targets we report here are therefore regulated differently indicating that OryR can probably function with, and without, the RSM and act as a positive as well as a negative transcriptional regulator.

Quorum sensing in *Xanthomonas* has been associated with the DSF signalling molecule thus we were interested to determine whether OryR and DSF signalling were interconnected. We established that OryR was not involved in DSF production since the *Xoo oryR* mutant was not altered in DSF synthesis and *rpfF* promoter activity. Furthermore, we determined that DSF quantities did not change in the presence of macerated rice. We cannot exclude the possibility that DSF can regulate *oryR*/OryR levels. However, a recent genome scale analysis in *Xcc* revealed

that DSF QS is not involved in the regulation of *xccR* (He *et al.*, 2006). It is therefore reasonable to assume that probably also in *Xoo,* DSF signalling is not regulating *oryR*. DSF cell-cell communication and OryR-RSM regulation are therefore acting independently and are not interconnected; it cannot be excluded however that the two systems might have overlapping regulons. Experiments reported here have also shown that the RSM is very small and does not interfere and/or act as an agonist in AHL QS systems. This suggest that this member of the LuxR-family, regardless of the conservation with AHL QS members does not bind AHLs, but an unknown RSM and is involved in inter-kingdom signalling.

3.5 EXPERIMENTAL PROCEDURES

3.5.1 Bacterial strains, media and growth conditions

Xanthomonas oryzae pv. *oryzae* strain XKK.12 was grown at 28 °C in PYS liquid medium (Ferluga *et al.*, 2007), PS (Tsuchiya *et al.*, 1982) solid medium and M9 minimal medium (Sambrook *et al.*, 1989) with the addition of casamino acids. *Escherichia coli* DH5α (Sambrook *et al.*, 1989) was grown at 37°C in Luria-Bertani (LB) medium (Miller, 1972), *A. tumefaciens* NT1(pZLQR) was grown at 28 °C in AB minimal medium (Cha *et al.*, 1998). Rice medium and infected rice medium were prepared by macerating healthy and infected rice plants (cv. Baldo) respectively (2,5% w/v), frozen in liquid nitrogen, the powder obtained was added to water, autoclaved for sterilization and filtered (Millipore) to remove rice tissue (Ferluga *et al.*, 2007). PYS-Rice was obtained by adding uninfected rice powder to PYS. When necessary, antibiotics were added at the following concentrations: ampicillin

(100 μg/ml), kanamycin (50 μg/ml) and gentamicin (30 μg/ml). Infected rice medium was fractionated by ultrafiltration using YM10, YM3 and YM1 membranes (Amicon Inc.). AHLs were acquired from the laboratory of Professor Paul Williams (University of Nottingham, UK).

3.5.2 Recombinant DNA techniques

DNA manipulations, including digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 ligase and transformation of *E. coli*, were performed as described previously (Sambrook *et al.*, 1989).

Table 1: *Xanthomonas oryzae* pv. *oryzae* strains, plasmids and oligonucleotides used in this study.

Strains, plasmids and oligonucleotides	Characteristics or sequence	Reference or source
Strains		
Xoo strain XKK.12	Wild type strain	(Ferluga *et al.*, 2007)
Xoo XKK.12ORYR	*Xoo* strain XKK.12 – OryR mutant	(Ferluga *et al.*, 2007)
A. tumefaciens NTL4(pZLQR)	Indicator strain for AHLs detection	(Cha *et al.*, 1998)
Plasmids		
pMOSBlue	Cloning vector, Amp^r	Amersham-Pharmacia
pSS122	Promoter probe vector, IncW, Ap^r-Gm^r	This study
pORY122	*oryR* promoter cloned *HindIII.SmaI* in pSS122	This study
pPIP122	*pip* promoter cloned *HindIII.PstI* in pSS122	This study
pCBS122	*cbs* promoter cloned *HindIII.SalI* in pSS122	This study
pRPFF122	*rpfF* promoter cloned *PstI.SalI* in pSS122	This study
Oligonucleotides		
UIDAS	5'- CCGGTACCTTGACCAGTATTAT -3'	This study
UIDAR	5'- CAGAATTCTCATTGTTTGCCTC -3'	This study
ORYPRS	5'- ATAAGCTTAGACGCCGCCGAAG -3'	This study
ORYPRR	5'- ATCCCGGGTAGACCAACGACTG -3'	This study
PIPPRS	5'- TTAAGCTTCGCGTGATGCGCTTG -3'	This study
PIPPRR	5'- TTCTGCAGTGGCCGCCAGATCCT -3'	This study
CBSPRS	5'- TTAAGCTTGCGTGTGGGCGTCAG -3'	This study
CBSPRR	5'- TTGTCGACCGCGCCTGTCAGCAA -3'	This study
RPFFPRS	5'- AAC TGC AGA TCG CCA CCA TGC -3'	This study
RPFFPRR	5'- CAG TCG ACC GTC GAA TTC TAT -3'	This study

Plasmids were purified using Jet star columns (Genomed GmbH, Löhne, Germany) or by he alkaline lysis method (Birnboim, 1983). Genomic DNA from *Xoo* was isolated

by Sarkosyl-pronase lysis as previously described (Birnboim, 1983). *Xoo* promoters were amplified by PCR and cloned in pMOSBlue cloning vector (Amersham-Pharmacia). All DNA sequencing was performed by Macrogen (www.macrogen.com). Reporter plasmid pSS122 was transferred to *Xoo* cells by electroporation as previously described (do Amaral *et al.*, 2005).

3.5.3 pSS122 promoter-probe plasmid construction

Plasmid pSS122 (IncW replicon) was constructed from pUFR047 (De Feyter *et al.*, 1993), a stably maintained plasmid at low copy number in both *E. coli* and *Xanthomonas*. Reporter gene *uidA* was amplified from *E. coli* K12 genomic DNA by PCR using UIDAS and UIDAR primers and cloned *Kpn*I-*Eco*RI in pUFR047. The resulting plasmid, approximately 10.5 Kb in size, has unique restriction sites for *Hin*dIII, *Pst*I, *Sal*I, *Sma*I and *Kpn*I. pSS122 retained ampicillin and gentamicin resistance genes but lost *lacZ*$^+$ marker.

3.5.4 β-glucuronidase assay

Overnight cultures of *Xoo*-pSS122 reporter plasmid carrying different promoters were assayed for β-glucuronidase activity. *Xoo* cells were pelleted and resuspended in 600 µl of GUS buffer (50 mM sodium phosphate (pH 7.0), 1 mM EDTA and 14.3 mM 2-mercaptoethanol). 23 µl of both TRITON X-100 3% in GUS buffer and sodium lauryl- sarcosinate 3% in GUS buffer were added to the samples and placed at 30°C for 10 minutes. 100 µl of *p*-nitrophenyl-β-D-glucuronic acid (PNPG) (Sigma) 25 mM were added. The reaction was stopped by adding 280 µl of 1M Na_2CO_3 solution after

sufficient yellow colour had developed. Both OD_{595} of *Xoo* cultures and $OD_{415\ PNPG}$ of the samples were measured and Miller Units (M.U.) of β-glucuronidase activity were determined as follows: 1 M.U.= 1000 x {[$OD_{415\ PNPG}$ – (1,75 x $OD_{595\ PNPG}$)] / (t x v x OD_{595})}, where t is the time of the reaction in minutes, v is the volume of the culture assayed in millilitres, OD_{595} is the cell density just before the assay, $OD_{415\ PNPG}$ is the measure of the yellow colour developed after the β-glucuronidase reaction, and 1,75 is the correction factor. All measurements were done at least in triplicate.

3.5.5 Rice infection and xylem sap collection

Xoo XKK.12 was grown on PS plates (Tsuchiya *et al.*, 1982) at 28 °C and single colonies were transferred to liquid PYS medium (Ferluga *et al.*, 2007). One-day-old culture adjusted to 10^9 CFU/ml was used to inoculate 6 weeks-old rice plants (cultivar IR24) by the clipping method as previously described (Ferluga *et al.*, 2007). To collect xylem sap, infected plants were placed in humid chambers and the dried blighted part of the infected leaf was removed cutting approximately 2-3 cm below the lesion. Drops from the xylem were collected during the subsequent 8 hours and placed in sterile tubes.

3.5.6 OryR over-expression and Western blot analysis

E. coli M15-pQEORYR (Ferluga *et al.*, 2007) was grown in 10 ml of LB medium adding 20 µl of xylem sap collected from *Xoo* infected rice plants. OryR expression was induced with 1mM of isopropyl-β-D-thiogalactoside at an OD_{600} of 0.6 and carried on for 1 h at 28°C. The culture was rapidly chilled on ice and soluble His_6-

OryR was extracted under native conditions according to the supplier's instructions (Qiagen). Proteins were transferred onto PVDF membrane (Immobilon-P; Millipore) using a tank system according to the manufacturer's instruction. The membrane was subjected to Western blot analysis using 6x-His monoclonal antibody (BD Biosciences, San Josè, CA, USA) and after incubation with the second HRP-labelled antibody the protein was detected with the 3-3'-diaminobenzidine (DAB) tetrahydrochloride (Sigma, St. Louis, Missouri,USA).

3.5.7 DSF measurements

DSF signalling regulates the production of protease and endoglucanase in *Xcc* (Barber *et al.*, 1997). Protease activity of *Xoo* XKK.12 parental strain and *Xoo* XKK.12ORY were assayed on skimmed milk plates as previously described (Barber *et al.*, 1997). Endoglucanase activity was visualized on CMC agar plates due to the ability of crude DSF extracts from *Xoo* to restore DSF production of *Xcc* indicator strain (*rpfF* mutant) (Barber *et al.*, 1997). *Xoo* XKK.12 and *Xoo* XKK.12ORY were grown in PYS rich medium, in macerated rice medium and in infected macerated rice medium. DSF was extracted from different culture volumes in order to normalize the number of cells of XKK.12 compared to *Xoo* XKK.12ORY for each medium.

3.5.8 Statistical analysis

P values were calculated using the ANOVA one way test led by MS excel. P values $\leq$0,05 were considered to be statistically significant.

3.6 ACKNOWLEDGEMENTS

S.F. is supported by an ICGEB fellowship. The research activities at the Biosafety Outstation are also supported by the Fondazione Cassamarca, Treviso, Italy. We wish to thank J.M. Dow for providing the DSF biosensor strain, Sujatha Subramoni for reading the manuscript and for fruitful discussions and Mirco Boem for seeding and growing the rice plants.

3.7 REFERENCES

Barber, C.E., Tang, J.L., Feng, J.X., Pan, M.Q., Wilson, T.J., Slater, H., Dow, J.M., Williams, P., and Daniels, M.J. (1997) A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol Microbiol* **24**: 555-566.

Bauer, W.D., and Mathesius, U. (2004) Plant responses to bacterial quorum sensing signals. *Curr Opin Plant Biol* **7**: 429-433.

Birnboim, H.C. (1983) A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol* **100**: 243-255.

Camara, M., Williams, P., and Hardman, A. (2002) Controlling infection by tuning in and turning down the volume of bacterial small-talk. *Lancet Infect Dis* **2**: 667-676.

Cha, C., Gao, P., Chen, Y.C., Shaw, P.D., and Farrand, S.K. (1998) Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. *Mol Plant Microbe Interact* **11**: 1119-1129.

Chatterjee, S., and Sonti, R.V. (2002) *rpfF* mutants of *Xanthomonas oryzae* pv. *oryzae* are deficient for virulence and growth under low iron conditions. *Mol Plant Microbe Interact* **15**: 463-471.

Cho, H.J., Park, Y.J., Noh, T.H., Kim, Y.T., Kim, J.G., Song, E.S., Lee, D.H., and Lee, B.M. (2007) Molecular analysis of the *hrp* gene cluster in *Xanthomonas oryzae* pathovar *oryzae* KACC10859. *Microbial Pathogenesis* Available online 23 December 2007.

De Feyter, R., Yang, Y., and Gabriel, D.W. (1993) Gene-for-genes interactions between cotton R genes and *Xanthomonas campestris* pv. *malvacearum* avr genes. *Mol Plant Microbe Interact* **6**: 225-237.

Degrassi, G., Devescovi, G., Solis, R., Steindler, L., and Venturi, V. (2007) *Oryza sativa* rice plants contain molecules that activate different quorum-sensing *N*-acyl homoserine lactone biosensors and are sensitive to the specific AiiA lactonase. *FEMS Microbiol Lett* **269**: 213-220.

Dixon, R.A. (2001) Natural products and plant disease resistance. *Nature* **411**: 843-847.

do Amaral, A.M., Toledo, C.P., Baptista, J.C., and Machado, M.A. (2005) Transformation of *Xanthomonas axonopodis* pv. *citri* by Electroporation. *Fitopatol. bras.* **30**: 292-294.

Dow, J.M., Crossman, L., Findlay, K., He, Y.Q., Feng, J.X., and Tang, J.L. (2003) Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. *Proc Natl Acad Sci U S A* **100**: 10995-11000.

Ferluga, S., Bigirimana, J., Höfte, M., and Venturi, V. (2007) A LuxR homologue of *Xanthomonas oryzae* pv. *oryzae* is required for optimal rice virulence. *Molecular Plant Pathology* **8**: 529-538.

Fuqua, C., Parsek, M.R., and Greenberg, E.P. (2001) Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* **35**: 439-468.

Fuqua, C. (2006) The QscR quorum-sensing regulon of *Pseudomonas aeruginosa*: an orphan claims its identity. *J Bacteriol* **188**: 3169-3171.

Givskov, M., de Nys, R., Manefield, M., Gram, L., Maximilien, R., Eberl, L., Molin, S., Steinberg, P.D., and Kjelleberg, S. (1996) Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. *J Bacteriol* **178**: 6618-6622.

He, Y.W., Wang, C., Zhou, L., Song, H., Dow, J.M., and Zhang, L.H. (2006) Dual signaling functions of the hybrid sensor kinase RpfC of *Xanthomonas campestris* involve either phosphorelay or receiver domain-protein interaction. *J Biol Chem* **281**: 33414-33421.

Jha, G., Rajeshwari, R., and Sonti, R.V. (2007) Functional interplay between two *Xanthomonas oryzae* pv. *oryzae* secretion systems in modulating virulence on rice. *Mol Plant Microbe Interact* **20**: 31-40.

Lee, S.W., Jeong, K.S., Han, S.W., Lee, S.E., Phee, B.K., Hahn, T.R., and Ronald, P. (2008) The *Xanthomonas oryzae* pv. *oryzae* PhoPQ two-component system is required for AvrXA21 activity, hrpG expression, and virulence. *J Bacteriol* **190**: 2183-2197.

Manefield, M., de Nys, R., Kumar, N., Read, R., Givskov, M., Steinberg, P., and Kjelleberg, S. (1999) Evidence that halogenated furanones from *Delisea pulchra* inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. *Microbiology* **145 (Pt 2)**: 283-291.

Miller, J.H. (1972) Experiments in molecular genetics. *Cold Spring Harbor Laboratory Cold Spring Harbor, N.Y.*

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*

Schuster, M., Urbanowski, M.L., and Greenberg, E.P. (2004) Promoter specificity in *Pseudomonas aeruginosa* quorum sensing revealed by DNA binding of purified LasR. *Proc Natl Acad Sci U S A* **101**: 15833-15839.

Shen, Y., and Ronald, P. (2002) Molecular determinants of disease and resistance in interactions of *Xanthomonas oryzae* pv. *oryzae* and rice. *Microbes Infect* **4**: 1361-1367.

Slater, H., Alvarez-Morales, A., Barber, C.E., Daniels, M.J., and Dow, J.M. (2000) A two-component system involving an HD-GYP domain protein links cell-cell signalling to pathogenicity gene expression in *Xanthomonas campestris*. *Mol Microbiol* **38**: 986-1003.

Swings, J., Van den Mooter, M., Vauterin, L., Hoste, B., Gillis, M., Mew, T.W., and Kersters, K. (1990) Reclassification of the causal agents of bacterial blight (*Xanthomonas campestris pv. oryzae*) and bacterial leaf streak (*Xanthomonas campestris pv. oryzicola*) of rice as pathovars of Xanthomonas oryzae (ex Ishiyama 1922) sp. nov., nom. rev. *Int. J. Syst. Bacteriol.* **40**: 309-311.

Teplitski, M., Chen, H., Rajamani, S., Gao, M., Merighi, M., Sayre, R.T., Robinson, J.B., Rolfe, B.G., and Bauer, W.D. (2004) *Chlamydomonas reinhardtii* secretes compounds that mimic bacterial signals and interfere with quorum sensing regulation in bacteria. *Plant Physiol* **134**: 137-146.

Tsuchiya, K., Mew, T.W., and Wakimoto, S. (1982) Bacteriological and. pathological characteristics of wild-types and induced mutants of *Xanthomonas campestris* pv. *oryzae*. *Phytopathology* **72**: 43-46.

Urbanowski, M.L., Lostroh, C.P., and Greenberg, E.P. (2004) Reversible acyl-homoserine lactone binding to purified *Vibrio fischeri* LuxR protein. *J Bacteriol* **186**: 631-637.

Vannini, A., Volpari, C., Gargioli, C., Muraglia, E., Cortese, R., De Francesco, R., Neddermann, P., and Marco, S.D. (2002) The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA. *Embo J* **21**: 4393-4401.

Walters, M., and Sperandio, V. (2006) Quorum sensing in *Escherichia coli* and *Salmonella*. *Int J Med Microbiol* **296**: 125-131.

Waters, C.M., and Bassler, B.L. (2005) Quorum sensing: cell-to-cell communication in bacteria. *Annu Rev Cell Dev Biol* **21**: 319-346.

Zhang, L., Jia, Y., Wang, L., and Fang, R. (2007) A proline iminopeptidase gene upregulated *in planta* by a LuxR homologue is essential for pathogenicity of *Xanthomonas campestris* pv. *campestris*. *Mol Microbiol* **65**: 121-136.

Zhang, R.G., Pappas, T., Brace, J.L., Miller, P.C., Oulmassov, T., Molyneaux, J.M., Anderson, J.C., Bashkin, J.K., Winans, S.C., and Joachimiak, A. (2002) Structure of a bacterial quorum-sensing transcription factor complexed with pheromone and DNA. *Nature* **417**: 971-974.

The plant opportunistic pathogen *Pseudomonas fuscovaginae* contains a conserved quorum sensing system involved in virulence

4.1 SUMMARY

Pseudomonas fuscovaginae is a fluorescent pseudomonad and an opportunistic plant pathogen of a wide variety of graminaceae, in particular it causes sheath rot in rice (*Oryza sativa*). This pathogen can cause severe problems especially in rice cultivation in highland fields 1200-1600 meters above sea level. In this study we have identified the *N*-acyl homoserine lactone (AHL) quorum sensing (QS) system and characterized its role in plant pathogenicity. *P. fuscovaginae* isolates from various areas of the world possess a conserved QS system, designated PfvI/R, responding to long chain-AHLs. This system displays significant similarity to the LasI/R system of *P. aeruginosa* and the PpuI/R system of *P. putida*. The PfvI/R system is involved in plant associated virulence in two plant models, ie. in rice and in *Chenopodium quinoa* which has never been reported previously. In addition, the PfvI/R system is required for eliciting a non-host plant response. This is the first report of a molecular study of virulence in this important plant opportunistic pathogen.

4.2 INTRODUCTION

Pseudomonas fuscovaginae is a Gram-negative fluorescent pseudomonad first identified and reported as a pathogen of rice (*Oryza sativa*) in Japan in 1976 (Miyajima *et al.*, 1983; Tanii *et al.*, 1976). Typical symptoms on mature rice plants are characterized by brown-black, water-soaked spots on the adaxial side of flag leaf sheath, with grain discoloration, poor spike emergence and sterility in severe cases (Duveiller *et al.*, 1989; Miyajima *et al.*, 1983; Zeigler and Alvarez, 1987). Symptoms on seedlings are limited to brown, water-soaked necrosis on the sheaths and can sometimes lead to death of the plant (Duveiller *et al.*, 1988; Rott and Notteghem, 1989; Zeigler, 1990). *P. fuscovaginae* is widespread and has been isolated from diseased rice in Latin America (Duveiller, 1990; Zeigler and Alvarez, 1987), Asia (GuanLin, 2003; Miyajima *et al.*, 1983) and Africa (Rott and Notteghem, 1989) in tropical upland fields, between 1450 and 2100 meters above sea level (Duveiller *et al.*, 1989). In fact, one distinctive feature is its ability to grow and colonize at high humidity and low temperatures, usually below 20 °C, even if the optimal growth temperature in the laboratory is 28°C (Miyajima *et al.*, 1983). *P. fuscovaginae* is now regarded as an opportunistic plant pathogen that causes bacterial brown sheath rot on several cereals including maize (*Zea mays*), sorghum (*Sorghum bicolor*) (Duveiller *et al.*, 1989) and wheat (*Triticum aestivum*) (Duveiller, 1990). Importantly, *P. fuscovaginae* can be distinguished from other fluorescent pseudomonads by a combination of biochemical tests, serological techniques and pathogenicity tests (Duveiller *et al.*, 1988; Rott, 1991).

The development of the disease and the progression of symptoms is believed to be associated with the production of several phytotoxins (Gross and Cody, 1985; Gross, 1991); thus far, three different types of phytotoxic metabolites, syringotoxin, fuscopeptin A (FP-A) and fuscopeptin B (FP-B) (Flamand *et al.*, 1996), were shown to be involved in generating the symptoms. Syringotoxin belongs to a group of antifungal metabolites known as lipodepsipeptides (LDPs) acting at the level of plasma membrane forming ion channels and consequently increasing membrane permeability (Batoko *et al.*, 1998; Hutchison *et al.*, 1995; Hutchison and Gross, 1997). FP-A and FP-B, equally characterized as LDPs (Ballio *et al.*, 1996), display similar toxic properties to syringotoxins and are structurally related to syringopeptins produced by pathogenic *P. syringae* pv. *syringae* strains (Ballio *et al.*, 1991). FP-A and FP-B have the same quantitative amino acid composition differing only for fatty acid moieties (Ballio *et al.*, 1996).

Despite the importance of *P. fuscovaginae* as an opportunistic pathogen on several plant hosts around the world, no molecular studies of virulence have thus far, to our knowledge, been reported. In this study we investigated the role of the quorum sensing gene regulatory system in sheath rot caused by *P. fuscovaginae* in rice. Quorum sensing (QS) is an intercellular communication system that couples bacterial cell density to gene expression via the production and detection of signal molecules (for reviews, see references: (Bassler, 1999; Fuqua *et al.*, 1994; Lazdunski *et al.*, 2004; Swift *et al.*, 1994; Zhang and Dong, 2004). In Gram-negative bacteria, *N*-acyl homoserine lactones (AHL) signal molecules are most commonly used; they are produced by an AHL synthase which in most cases belongs to the LuxI-protein

family. A transcriptional regulator belonging to the LuxR family then forms a complex with the cognate AHL at threshold ('quorum') concentration and affects the transcriptional status of target genes (Fuqua *et al.*, 2001). The *luxI* and *luxR* type genes are in most cases genetically linked and the system usually is under a positive feedback loop resulting in signal amplification at high cell density. QS-dependent regulation in bacteria is most often beneficial to a community of bacteria as for example is the case for biofilm formation, conjugation, bioluminescence, production of extracellular enzymes, virulence factors and pigment formation (Fuqua *et al.*, 2001; Whitehead *et al.*, 2001), as for example extracellular enzyme production in *Erwinia carotovora*, conjugation in *Agrobacterium tumefaciens* and toxin production in *Burkholderia glumae* (Kim *et al.*, 2004; Von Bodman *et al.*, 2003).

In this study we report the identification and characterization of the AHL QS system of the rice sheath rot pathogen *P. fuscovaginae*. We studied 15 *P. fuscovaginae* strains isolated from diseased rice from various parts of the world for their AHL production. The AHL QS system, designated PfvI/R, of two *P. fuscovaginae* strains has been isolated, characterized and found to be highly conserved also among all the other 13 *P. fuscovaginae* strains tested. The PfvI/R system displayed high similarity to the LasI/R and PpuI/R systems of *Pseudomonas aeruginosa* and *Pseudomonas putida* respectively (Bertani and Venturi, 2004; Venturi, 2006). AHL QS in *P. fuscovaginae* was shown to be important for sheath rot in rice and also in the convenient *Chenopodium quinoa-P. fuscovaginae* plant-virulence model which we report here. Finally, we also observed that the PfvI/R system of *P. fuscovaginae* was involved in

the hypersensitive response in non-host plants. This is the first report of a molecular study of virulence in this important plant opportunistic pathogen.

4.3 RESULTS AND DISCUSSION

4.3.1 AHL QS is well conserved in *P. fuscovaginae*

In order to determine if *P. fuscovaginae* could synthesize AHLs, we analysed a collection of 15 different strains for AHLs production; all these strains were isolated from diseased *Oryza sativa* grown in very different geographical regions (Table 2). Thirteen of these strains are able to cause bacterial brown sheath rot on rice plant whereas the two Colombian strains (UPB0898 and LMG12428) appeared to cause leaf stripe (unpublished; Zeigler and Notteghem personal communication). The strains were initially tested for AHL production by a plate T-streak using biosensors CV026 and F117 (pRKC12) as described in the Material and Methods section. CV026 is a specific sensor to detect short and medium acyl chain AHLs whereas F117(pRKC12) is sensitive to C_{12}-3oxo- and C_{10}-3oxo-AHL (Steindler and Venturi, 2007). It was observed that only strains UPB0898 and LMG12428 were able to induce a strong activation of both biosensors on plates, probably indicating high levels of AHLs. We then decided to examine AHL production in all the 15 strains by purifying AHLs from culture supernatants and performing TLC analysis. Due to its capability to respond to a wide range of AHL molecules, *A. tumefaciens* NTL4 (pZLR4) was used as biosensor for this experiment (Figure 1).

Three strains, LMG5742, UPB0898, and LMG12428, displayed the same profile probably producing C_6-3oxo-AHL, C_8-3oxo-AHL, C_{10}-3oxo-AHL, C_{12}-3oxo-AHL.

The other strains appeared to produce C_8-3oxo-AHL and C_{10}-3oxo-AHL since the retention factor (Rf) of these spots was similar to the Rf of synthetic AHLs.

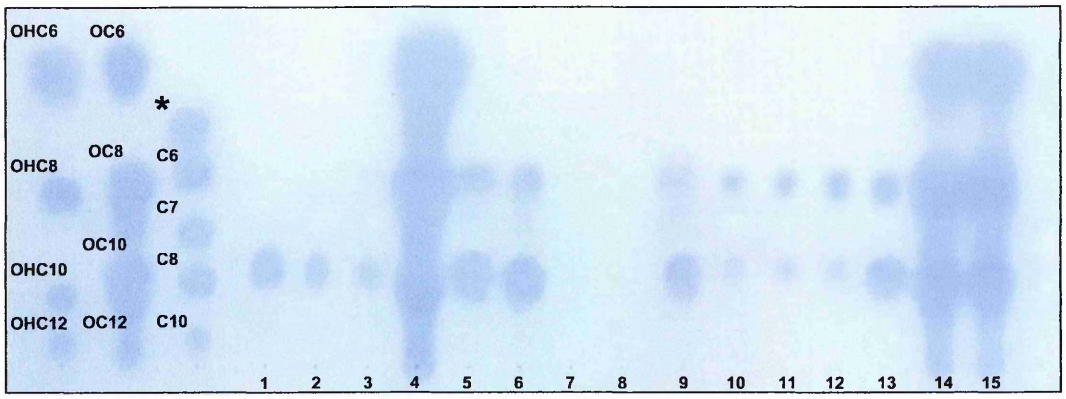


Figure 1: TLC analysis representing AHL production profile of the 15 *P. fuscovaginae* strains using *A. tumefaciens* NTL4 (pZLR4) as overlay biosensor. Synthetic AHLs were used to compare the retention factor (Rf) of the unknown AHLs produced by *P. fuscovaginae* (hydroxy substituted AHLs, first lane; oxo substituted AHLs, second lane; unsubstituted AHLs, third lane). Unsubstituted C_4- and C_{12}-AHL are not detectable and the asterisk marks an unknown active component (Shaw *et al.*, 1997). Numbers correspond to the following strains (see Table 1 and Table 2 for details): 1, LMG2158^T; 2, LMG2192; 3, LMG5097; 4, LMG5742; 5, LMG12424; 6, LMG12425; 7, UPB0266; 8, UPB0266b; 9, UPB0304; 10, UPB0305; 11, UPB0306; 12, UPB0306b; 13, UPB0736; 14, UPB0898; 15, LMG12428.

It was concluded that all strains had a similar AHL production profile and that three strains produced considerably greater amounts of AHLs indicating that AHL QS is present and the system(s) might be conserved within the species.

4.3.2 Identification and characterization of the AHL QS systems of *P. fuscovaginae* LMG12428 and UPB0736

Following the observation that all the strains tested produced similar AHLs, it was of interest to identify and characterize the AHL QS system of *P. fuscovaginae* in two strains which were reported to display different symptoms in rice. In order to identify the AHL-QS locus in *P. fuscovaginae* LMG12428, we constructed a cosmid library of this strain and then screened it by complementation in *trans* in CV026 AHL biosensor

as described in the Materials and Methods section. A cosmid clone able to restore purple pigmentation of CV026 was isolated and shown to contain the typical *luxI*-

Table 1: *Pseudomonas fuscovaginae* strains, plasmids and oligonucleotides used in this study.

Strains, plasmids and oligonucleotides	Characteristics or sequence	Reference or source
Strains		
P.fuscovaginae LMG 2158^T	Wild type strain	LMG Collection
P.fuscovaginae LMG 2192	Wild type strain	LMG Collection
P.fuscovaginae LMG 5097	Wild type strain	LMG Collection
P.fuscovaginae LMG 5742	Wild type strain	LMG Collection
P.fuscovaginae LMG 12424	Wild type strain	LMG Collection
P.fuscovaginae LMG 12425	Wild type strain	LMG Collection
P.fuscovaginae UPB 0266	Wild type strain	H. Maraite
P.fuscovaginae UPB 0266 b	Wild type strain	H. Maraite
P.fuscovaginae UPB 0304	Wild type strain	H. Maraite
P.fuscovaginae UPB 0305	Wild type strain	H. Maraite
P.fuscovaginae UPB 0306	Wild type strain	H. Maraite
P.fuscovaginae UPB 0306 b	Wild type strain	H. Maraite
P.fuscovaginae UPB 0736	Wild type strain	H. Maraite
P.fuscovaginae UPB 0898	Wild type strain	H. Maraite
P.fuscovaginae LMG 12428	Wild type strain	LMG Collection
P.fuscovaginae 13R	*P.fuscovaginae* strain UPB0736 - *pfvR* mutant	This study
Plasmids		
pBluescriptKS	Cloning vector, Amp^r	Stratagene
pMOSBlue	Cloning vector, Amp^r	Amersham-Pharm.
pQE30	Expression vector, Amp^r	Qiagen
pRK2013	Km^r Tra$^+$ Mob$^+$ ColE1 replicon	(Figurski, 1979)
pMP220	Promoter probe vector, IncP1, Tc^r	(Spaink, 1987)
pKNOCK-Km	Suicide vector for gene knockout, Km^r	(Alexeyev, 1999)
pLAFR3	Broad-host-range cloning vector IncP1, Tc^r	(Staskawicz 1987)
pSG100	pLAFR3 cosmid containing the QS locus of LMG12428	This study
pSG100L	QS locus of LMG12428 subcloned in pBluescriptKS	This study
pSG120	QS locus of UPB0736 cloned in pBluescriptKS	This study
pKNR13	*pfvR*-internal fragment of UPB0736 cloned in pKNOCK-Km	This study
pQER13	*pfvR* of UPB0736 cloned in pQE30	This study
pQER15	*pfvR* of LMG12428 cloned in pQE30	This study
pMPI13	*pfvI* promoter of UPB0736 cloned in pMP220	This study
pMPI15	*pfvI* promoter of LMG12428 cloned in pMP220	This study
Oligonucleotides		
PFVR15F	5'- GCGGATCCCTACTTATGGATGA -3'	This study
PFVR15R	5'- GAAAGCTTCTAGGGCGTCATGA -3'	This study
IPR15F	5'- CCGAATTCCTCAACCCGAACAT -3'	This study
IPR15R	5'- CATCTAGAGTCCTGCAGGATCA -3'	This study
PFVR13F	5'- TCGGATCCACCCTATGGTAA -3'	This study
PFVR13R	5'- GTAAGCTTTCAGGGCGTGATC -3'	This study
IPR13F	5'- TTGGTACCCGACCGCTGAGGTA -3'	This study
IPR13R	5'- GATCTAGATTCAGCGCTTGCGG -3'	This study
PK13RF	5'- ACTCTAGACTGATCGGTCTCAA -3'	This study
PK13RR	5'- AACTCGAGTGCTGCGCTCCACT -3'	This study

luxR family genes; the AHL-QS system of *P. fuscovaginae* was designated as *pfvI/R* (Figure 2-A). The PfvI/R system displayed significant similarity to the PpuI/R and LasI/R systems of *P. putida* and *P. aeruginosa* respectively (see below); these two systems produce and respond to C_{12}-3oxo-AHL (Bertani and Venturi, 2004; Pearson *et al.*, 1995; Pesci *et al.*, 1997).

Table 2: Geographical and biological origin of the 15 *P. fuscovaginae* strains used in this study and possible acyl homoserine lactone (AHL) molecules produced. The symbol (-) indicates no biosensor induction whereas the symbol (+) indicates the induction of the biosensor; the number of (+) refers to the intensity of the response.

Number	Strain	Geographic origin	Biological origin	NTL-4	PKRC12	CV026
1	LMG 2158^T	Japan	Oryza sativa	OC10	+	-
2	LMG 2192	Japan	Oryza sativa	OC10	+	-
3	LMG 5097	Japan	Oryza sativa	OC10	+	-
4	LMG 5742	Burundi	Oryza sativa	OC6-OC8-OC10-OC12	++	+
5	LMG 12424	Philippines	Oryza sativa	OC8-OC10	+	-
6	LMG 12425	Philippines	Oryza sativa	OC8-OC10	+	-
7	UPB 0266	Burundi	Oryza sativa	-	-	-
8	UPB 0266 b	Burundi	Oryza sativa	-	-	-
9	UPB 0304	Japan	Oryza sativa	OC8-OC10	+	-
10	UPB 0305	Japan	Oryza sativa	OC8-OC10	+	-
11	UPB 0306	Japan	Oryza sativa	OC8-OC10	+	-
12	UPB 0306 b	Japan	Oryza sativa	OC8-OC10	+	-
13	UPB 0736	Madagascar	Oryza sativa	OC8-OC10	+	-
14	UPB 0898	Colombia	Oryza sativa	OC6-OC8-OC10-OC12	+++	+++
15	LMG 12428	Colombia	Oryza sativa	OC6-OC8-OC10-OC12	+++	+++

In between the *pfvI/R* genes was located *rsaL* (Figure 2-A), which in *P. aeruginosa* and *P. putida* encodes a negative regulator that was shown to negatively regulate the AHL synthase gene (de Kievit *et al.*, 1999; Rampioni *et al.*, 2006). In order to identify the AHL-QS locus in *P. fuscovaginae* UPB0736, the *pfvI* gene of strain LMG12428 was used as a probe and a plasmid clone, designated pSG120, was identified containing the *pfvI/R* system of strain UPB0736 as described in the Materials and Methods. The AHL QS system of strain UPB0736 was similar to the one of strain LMG12428 having the genes *pfvR-rsaL-pfvI* arranged in a similar way (Figure 2-A).

The two *pfvI* genes (546 bp- *pfvI* LMG12428 and 543 bp- *pfvI* UPB0736) encoded two proteins of 181 and 180 amino acids long respectively, having an identity of 47.2% (similarity 57.3%).

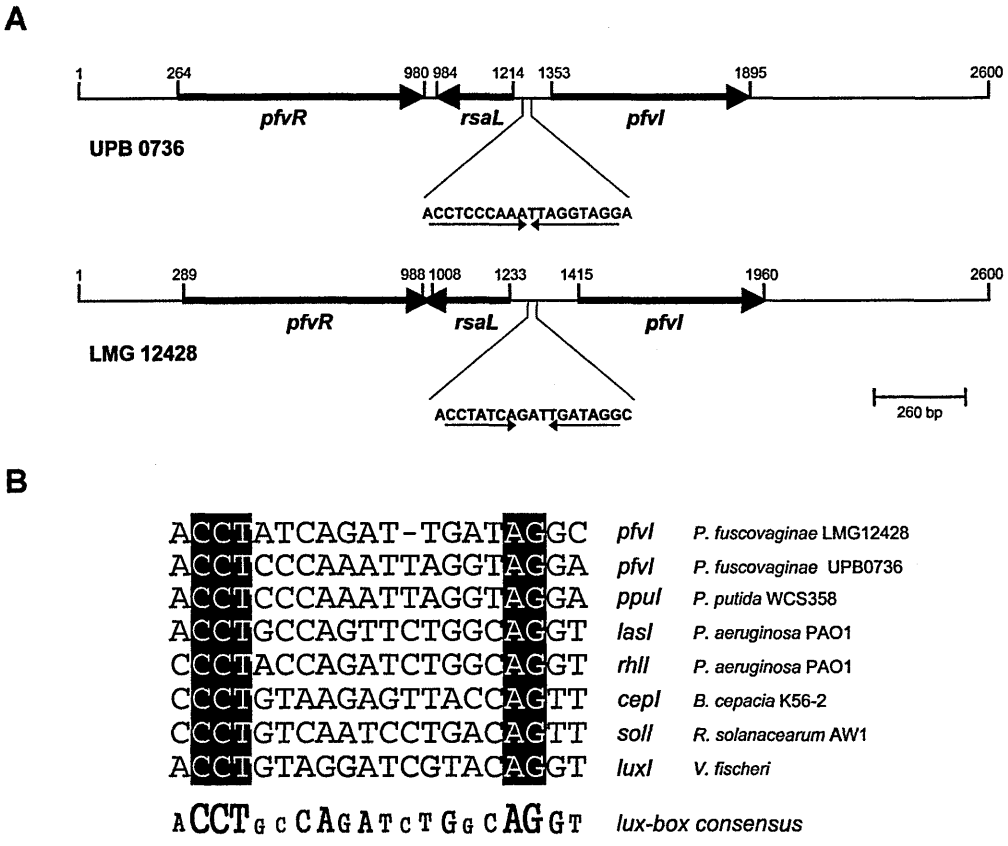


Figure 2: (A) Quorum sensing locus gene organization in *P.fuscovaginae* strain UPB0736 and strain LMG12428. For both strains a putative *lux*-box was identified in the *pfvI* promoter region. (B) Alignment of the putative *lux*-boxes of *P. fuscovaginae* strain UPB0736 and strain LMG12428 with other known *lux* boxes and with the *lux*-box consensus sequence. The most conserved nucleotides are shown in bold.

PfvI-LMG12428 was shown to be 54% identical to PpuI of *P. putida* and 47.8% identical to LasI of *P. aeruginosa* whereas PfvI-UPB 0736 was 81.7% identical to PpuI and 47.3% identical to LasI. The two PfvR proteins (encoded by 720 bp-*pfvR* LMG12428 and by 717 bp- *pfvR* UPB0736) were 43.8% identical and 58.3% similar

and both displayed high identity to PpuR of *P.putida* strains and to LasR of *P.aeruginosa*. Interestingly, PfvR-UPB0736 was 99.2% identical to PpuR (PfvR-LMG12428 identity was 43.2%) and 42.7% identical to LasR (pfvR-LMG12428 identity was 40.6%).

The RsaL-LMG12428 and RsaL-UPB0736 display an identity of 33.7% (48.8% similarity) however RsaL-UPB0736 was 100% identical to RsaL of *P. putida* (RsaL-LMG12428 was 33.7% identical) whereas was only 36.2% identical to the RsaL of *P. aeruginosa* strain (RsaL-LMG12428 was 27.3% identical). This data suggested that AHL-QS in *P. fuscovaginae* was more closely related to AHL-QS of *P. putida* than to AHL-QS of *P. aeruginosa*, especially for strain UPB0736, in which not only PfvR and RsaL proteins were identical but also the intergenic DNA region of the AHL QS genes was absolutely conserved. In the *pfvI* promoter regions of strain LMG12428 and strain UPB0736, putative *lux*-boxes, probably important for the binding and regulation of PfvR, were found (Figure2-A). The putative *lux*-box of *P. fuscovaginae* UPB0736 was identical to the mapped and experimentally shown *lux*-box of *P. putida* WCS358 (Rampioni *et al.*, 2006), whereas the *lux*-box of *P. fuscovaginae* LMG12428 also displayed very high identity but contained a deletion of a single nucleotide (Figure 2-B).

4.3.3 The PfvI/R AHL QS system is very well conserved in *P. fuscovaginae*

In order to determine the conservation of the PfvI/R AHL QS system among *P. fuscovaginae* strains, a Southern blot analysis was performed on the chromosomal DNA of the 15 isolates reported in Table 2, using the *pfvI* gene of *P. fuscovaginae* strain LMG12428 as probe. The experiment was performed at high hybridization

stringency conditions giving a strong positive hybridization signal with 13 of the 15 isolates (Figure 3).

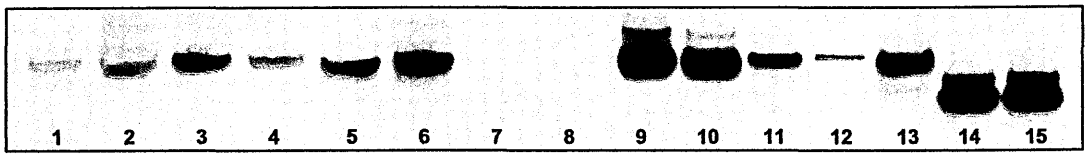


Figure 3: Southern blot analysis using *pfvI* DNA of strain LMG12428 as a probe against *Hin*cII-digested chromosomal DNA from 15 *P. fuscovaginae* strains. Numbers correspond to the following strains (see Table 1 and Table 2 for details): 1, LMG2158^T; 2, LMG2192; 3, LMG5097; 4, LMG5742; 5, LMG12424; 6, LMG12425; 7, UPB0266; 8, UPB0266b; 9, UPB0304; 10, UPB0305; 11, UPB0306; 12, UPB0306b; 13, UPB0736; 14, UPB0898; 15, LMG12428.

This data together, with similar TLC AHL producing profiles (see above), indicated that most *P. fuscovaginae* strains, if not all, isolated from geographically distant parts of the world, possess the PfvI/R system, suggesting that this AHL QS system was a feature of this species and thus it might play a central regulatory role.

4.3.4 Studies on PfvR-AHL specificity

LuxR family QS proteins bind the cognate AHL molecule(s) with high affinity most commonly resulting in the formation of homomultimers, resistant to proteases, and so becoming active regulatory molecules affecting transcription of target genes (Chai and Winans, 2004; Collins *et al.*, 2005; Schuster *et al.*, 2004; Urbanowski *et al.*, 2004; Zhu and Winans, 2001). To characterize PfvR AHL specificity, the protein was over-expressed in *E.coli* M15 in the presence of different AHL molecules and cognate *pfvI* promoter activities were then determined. Both gene promoters *pfvI*-UPB0736 and *pfvI*-LMG12428 were therefore cloned in the broad-host-range, low-copy-number β-galactosidase promoter probe vector pMP220 (Spaink *et al.*, 1987), yielding pMPI-13

and pMPI-15 vectors respectively and introduced into *E. coli* M15 expressing the cognate PfvR protein, generating *E. coli* M15 (pQER13)(pMPI-13) and *E. coli* M15 (pQER15)(pMPI15). The activity of the *pfvI*-UPB0736 promoter increased fourfold in the presence of C_{10}-3oxo-AHL and C_{12}-3oxo-AHL and about threefold in the presence of C_8-3oxo-AHL, C_{12}-AHL, C_{10}-AHL, C_{12}-3OH-AHL and C_{10}-3OH-AHL, demonstrating a preference for long-chain AHLs (Figure 4). The same activity profile was observed for the *pfvI*-LMG12428 promoter, showing an increase of ten-times in the presence of C_{10}-3oxo-AHL and C_{12}-3oxo-AHL and an increase of about six/seven times in the presence of C_8-3oxo-AHL, C_{12}-AHL, C_{10}-AHL, C_{12}-3OH-AHL and C_{10}-3OH-AHL (Figure 4).

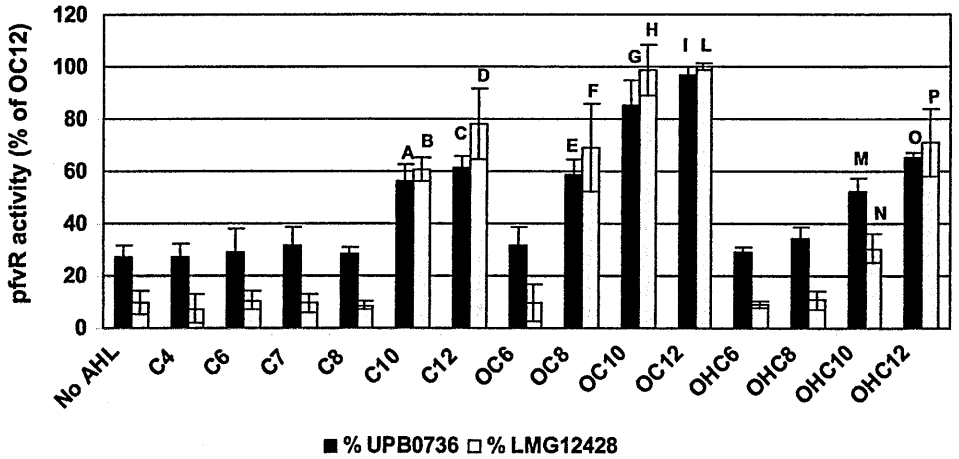


Figure 4: *pfvI* promoter activity of *P. fuscovaginae* strain UPB0736 and strain LMG12428 in the presence of different AHL molecules. The assay was performed in triplicate in *E. coli* M15 over-expressing *pfvR* (see text for details). PfvR in both strains responds best to C_{12}-3oxo-AHL and C_{10}-3oxo-AHL, showing however a relaxed specificity being able to respond also to C_{12}-AHL, C_{10}-AHL, C_8- 3oxo-AHL, C_{12}- 3OH-AHL and C_{10}- 3OH-AHL. The results are expressed as means ± STDEV, n=3. Statistically significant differences ($p \leq 0,05$) were analysed: A, $p < 0,02$; B, $p < 1,5 \times 10^{-4}$; C, $p < 4 \times 10^{-4}$; D, $p < 0,001$; E, $p < 0,001$; F, $p < 0,004$; G, $p < 6 \times 10^{-4}$; H, $p < 1,3 \times 10^{-4}$; I, $p < 2,64 \times 10^{-5}$; L, $p < 4,85 \times 10^{-6}$; M, $p < 0,002$; N, $p < 0,007$; O, $p < 10^{-4}$; P, $p < 0,001$, compared to the control without AHLs.

Interestingly, it was therefore concluded that PfvR had relaxed specificity towards AHLs being able to respond very well to two AHLs and reasonably well to a set of five other long-chain AHLs. This is in contrast to what has been reported for the similar PpuR and LasR proteins which were found more specific and responded well only to C_{12}-3oxo-AHL .

4.3.5 PfvR is involved in *P. fuscovaginae* pathogenicity *in planta*

To examine whether AHL-QS in *P. fuscovaginae* was involved in pathogenicity and in disease development, *P. fuscovaginae* UBB0736 parental strain and *P. fuscovaginae* 13R (*pfvR* knock-out mutant) were inoculated on *Chenopodium quinoa* and *Oryza sativa* host plants and disease development was evaluated.

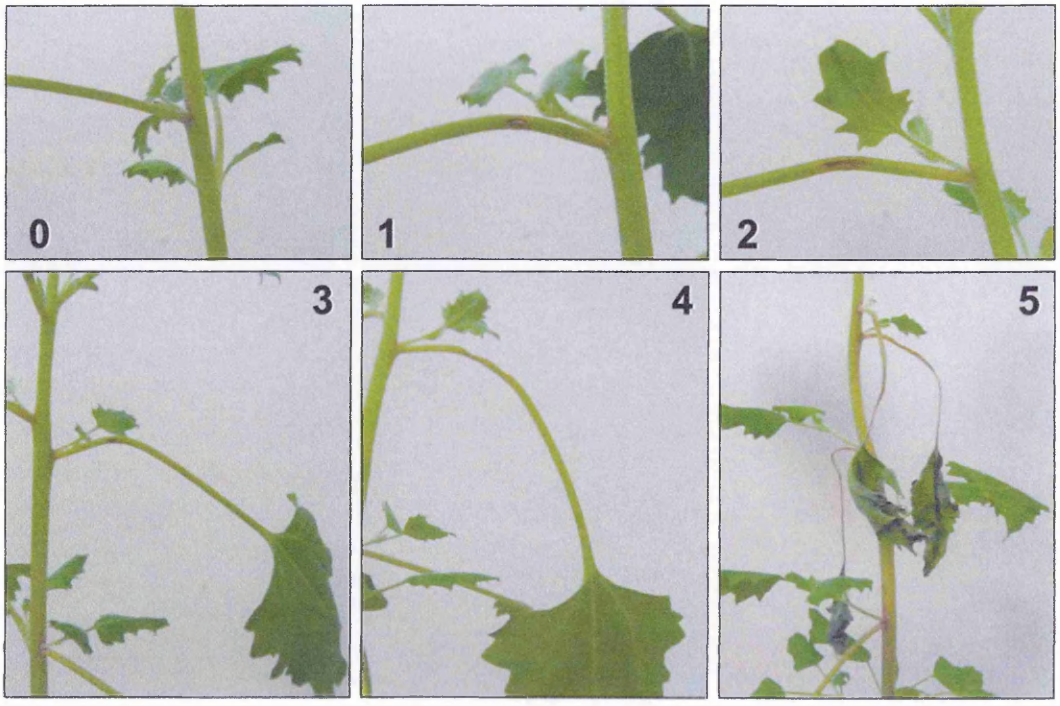


Figure 5: Severity scale used to evaluate disease caused by *P. fuscovaginae* infection on *Chenopodium quinoa*: 0, no symptoms; 1, necrosis on less than 2 mm around the puncture; 2, necrosis from 2 to 10 mm around the puncture; 3, necrosis from 2 to 10 mm around the puncture and bending of the petiole; 4, collapse of the petiole and 5, wilting of the leaf.

Here we report for the first time the use of *C. quinoa* as a plant model to assess *P. fuscovaginae* virulence (Figure 5 and Materials and Methods). Five days after infection (see Materials and Methods for details) disease development on *C. quinoa* and rice was evaluated with a 0-5 disease severity index as depicted in Figures 5 and 6.

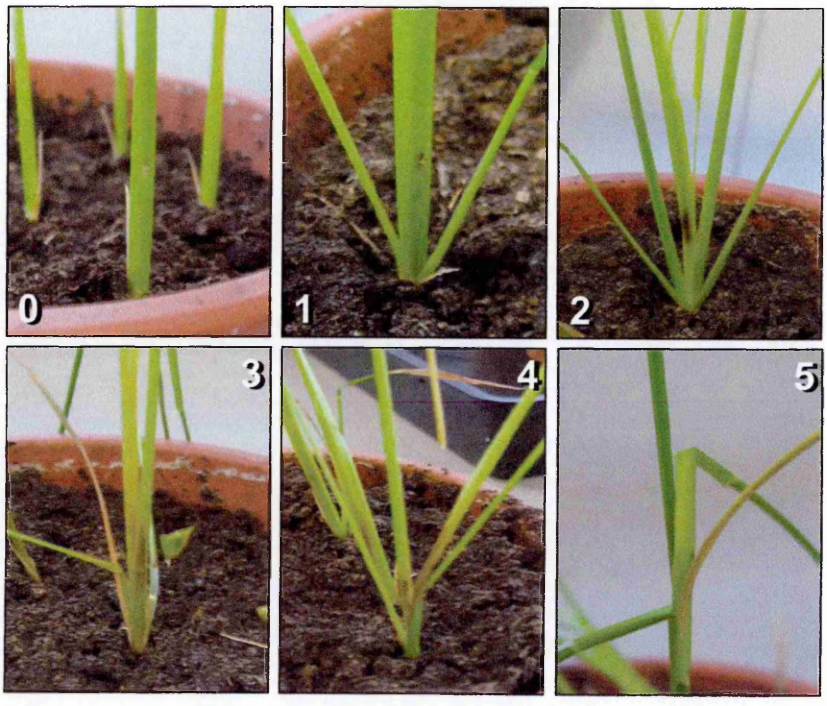


Figure 6: Severity scale used to evaluate disease caused by *P. fuscovaginae* infection on *Oryza sativa*: 0, no symptoms; 1, necrosis around the puncture till 1 cm; 2, necrosis around the puncture and chlorosis 1 to 3 cm on the new leaf; 3, necrosis around the puncture and chlorosis till 5 cm on the new leaf; 4, necrosis around the puncture and chlorosis for the two third of the new leaf and 5, necrosis around the puncture and chlorosis of all the new leaf.

P. fuscovaginae UBB0736 parental strain was very pathogenic on both *C. quinoa* and rice plants whereas *P. fuscovaginae* 13R was attenuated in virulence in both plants showing less severe disease index, as we can see from the results of the statistical analysis. The Kruskall-Wallis test for the *C. quinoa* gave a high score for the χ^2 test (20.828) and a very low score for the variance ($p<0.0001$); the Mann-Whitney test

showed a low score for the variance ($p<0.0001$). For the rice infection the results are almost the same: $\chi^2=15.436$ and $p<0.0001$ for the Kruskall-Wallis test and $p=0.005$ for the Mann-Whitney test.

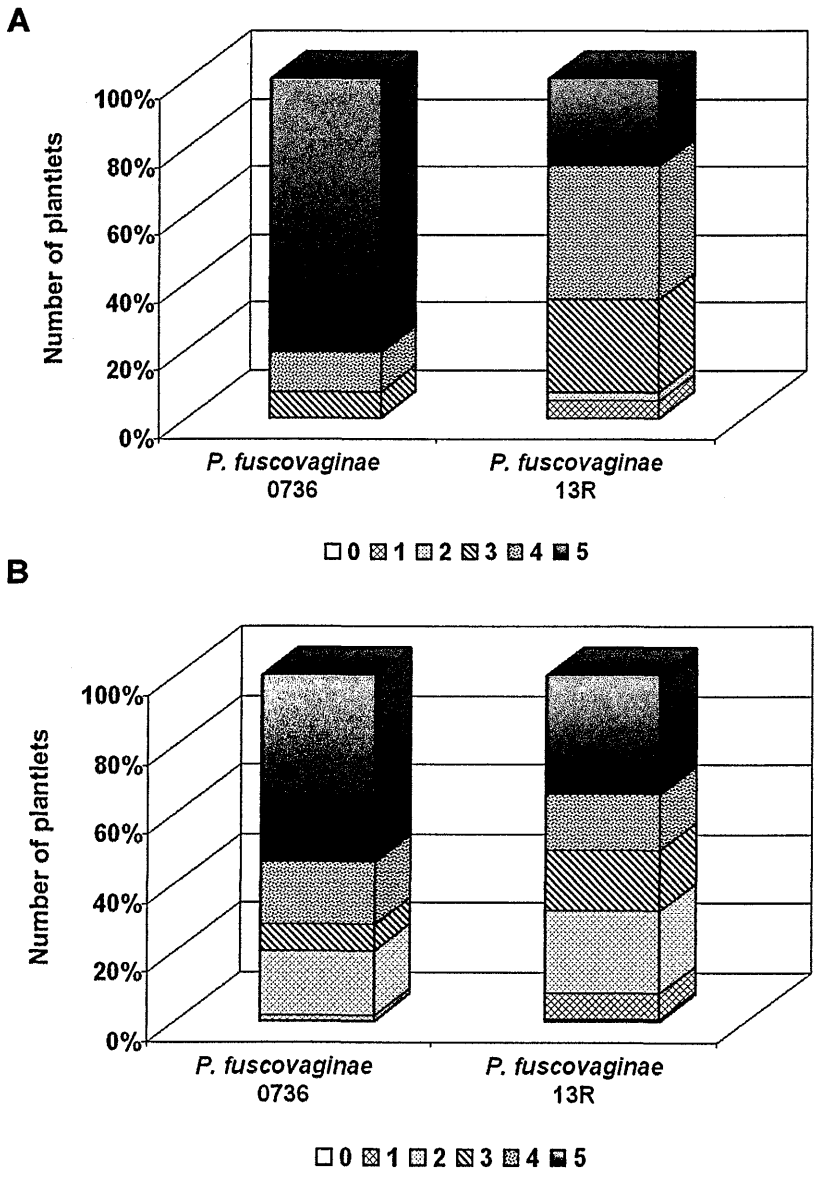


Figure 7: *P. fuscovaginae* UPB0736 parental strain and *P. fuscovaginae* 13R (*pfvR* mutant) pathogenicity assay performed on *Chenopodium quinoa* (A) and *Oryza sativa* (B). Disease was evaluated in agreement with a 0-5 severity scale (Figures 5-6). See text for statistical analysis details.

The ability to cause disease symptoms was only partially restored when *P. fuscovaginae* 13R was complemented *in trans* with pMP122 harbouring the *pfvR* gene because the plasmid was found to be highly unstable without antibiotic selection: just 1% of the cells recovered from infected plant tissue held the plasmid (data not shown). It is therefore evident that a functional AHL QS was essential for optimal *P. fuscovaginae* virulence on host plants suggesting that it is involved in the regulation of virulence associated factors. It is believed that symptoms of sheath brown rot caused by *P. fuscovaginae* are caused by the phytotoxins prior to extensive colonization. It is possible therefore that QS is involved in part in the regulation of expression of the phytotoxin structural genes; the role that QS might play in progressive plant colonization is currently unknown.

4.3.6 The *P. fuscovaginae pfvR* mutant is unable to cause HR on tobacco

It was previously reported that *P. fuscovaginae* is able to elicit a plant defence response in non-host plants (also known as the hypersensitive response or HR) like tobacco (Duveiller *et al.*, 1988). We therefore tested the ability of *P. fuscovaginae* UPB0736 parental strain to cause HR on tobacco (*Nicotiana tabacum*) leaves by infiltrating leaf mesophyll with bacterial suspensions (10^8 CFU mL^{-1}). It was determined that browning and tissue collapse was detectable at the injection sites indicating the development of a clear HR (Figure 8).

The quorum sensing mutant *P. fuscovaginae* 13R was also tested in order to establish the involvement of QS during this process. No detectable changes, just like the control, in the tissue structure were observed at the injection sites (Figure 8), indicating that *pfvR* and hence quorum sensing, is necessary to develop the HR

reaction on tobacco leaves. The necrotic phenotype which we observe in non-host tobacco could also be due to the phytotoxins produced by wild-type *P. fuscovaginae*; future work will precisely determine how this plant hypersensitive response occurs.

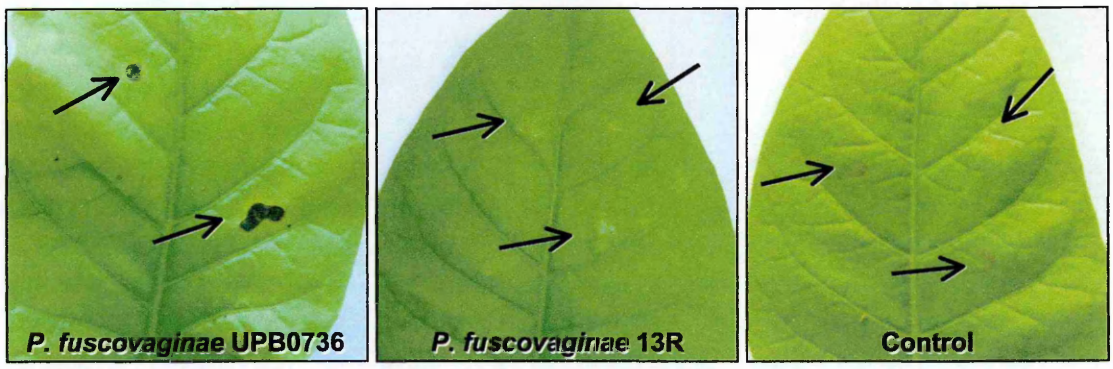


Figure 8: HR developed on tobacco leaves (*Nicotiana tabacum*) infiltrating leaf mesophyll with a bacterial suspension (10^8 CFU mL^{-1}) of *P. fuscovaginae* UPB0736 parental strain and *P. fuscovaginae* 13R, *pfvR* mutant. Sterile medium was infiltrated as control. The black arrows indicate the infiltration sites.

4.3.7 P. fuscovaginae motility and exoenzyme production

Bacteria can remain localized or can move to colonize larger areas depending on availability of nutrients and surface conditions. Swimming is mediated by polar flagella that enable bacteria to swim in aqueous environments or on semi-solid surfaces; swarming is the movement of a group of bacteria associated with the production of multiple lateral flagella and cell-cell contacts (Harshey, 2003).

To our knowledge no studies on the motility of *P. fuscovaginae* have been performed so far, therefore we tested the swimming and swarming activity of *P. fuscovaginae* strain UPB0736 in laboratory conditions (see Materials and Methods). To assess the involvement of QS in bacterial motility regulation we performed the assays also on *P. fuscovaginae*13R and on complemented strain *P. fuscovaginae* 13R-pMP122. *P. fuscovaginae* UPB0736 parental strain was able to swim, creating a dendrite, while *P.*

*fuscovaginae*13R (*pfvR* mutant) generated numerous dendrites and was able to swim considerably further (Figure 9). The *P. fuscovaginae* 13R-pMP122 complemented strain restored the parental phenotype.

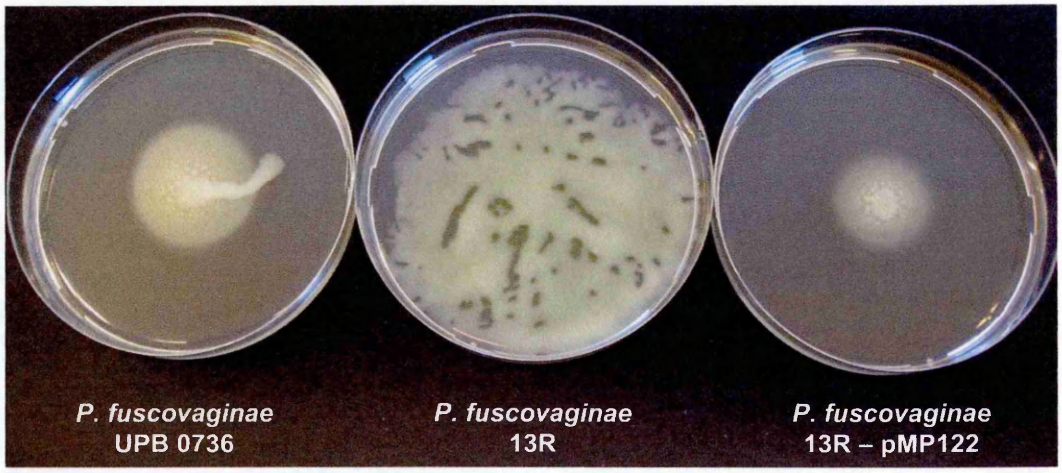


Figure 9: Swimming motility assay of *P. fuscovaginae* parental strain UPB0736 compared to the *P. fuscovaginae* 13R (*pfvR* mutant) and to *P. fuscovaginae* 13R complemented *in trans* with pMP122 containing *pfvR* gene.

These results indicated that possibly one or more polar flagella are present in *P. fuscovaginae* and that quorum sensing is involved in motility regulation.

P. fuscovaginae UPB0736 was unable to grow on M8 minimal medium thus we could not evaluate the swarming motility and role of quorum sensing. This result does not exclude that *P. fuscovaginae* is able to swarm but leaves the possibility that one or more essential nutrients, such essential amino acids, different carbon sources or different experimental conditions, are necessary for swarming (Kohler *et al.*, 2000). *P. fuscovaginae* UPB0736 did not display any proteolytic and lipolytic activity on plate assays in laboratory conditions (see Materials and Methods).

4.4 CONCLUDING REMARKS

This is the first genetic and molecular study of virulence of the opportunistic plant pathogen *P. fuscovaginae*. This bacterium is closely related to other fluorescent pseudomonads being widespread in many different geographical regions and having a wide host range among the graminaceae. In this study we have shown that distantly isolated strains possess a conserved AHL QS system and that it plays a role in plant associated virulence and hypersensitivity response in a non-host plant. *P. fuscovaginae* is an opportunistic plant pathogen requiring, in conjunction, a crop at the susceptible growth stage and a favourable environment for disease development (highland swamps 1300-1600 metres above sea level and low temperatures of approximately 20°C). Future studies will focus on the targets of the AHL QS related to plant infection, colonization and progression of the disease. In addition, studying *P. fuscovaginae* may reveal interesting differences and similarities to closely related fluorescent pseduomonads which are either plant beneficial bacteria or human opportunistic pathogens.

4.5 EXPERIMENTAL PROCEDURES

4.5.1 Bacterial strains, plasmids, and media

All strains and plasmids used in this study are listed in Table 1. *P. fuscovaginae* and derivatives were cultured at 30°C in King B medium (KB) (King *et al.*, 1954) and M9 minimal medium (Sambrook *et al.*, 1989) supplemented with 0.3% citric acid and 0.3% casamino acids. *Escherichia coli* DH5α (Sambrook *et al.*, 1989), *E. coli* DH5α (pRK2013) (Figurski and Helinski, 1979), *E .coli* HB101 (Magazin *et al.*, 1986) and *E. coli* JM109 (pSB401) (Winson *et al.*, 1998) were grown at 37°C in Luria-Bertani (LB) medium (Miller, 1972). *Chromobacterium violaceum* strain CVO26 (McClean *et al.*, 1997) and *Pseudomonas putida* F117 (pKRC12) (Riedel *et al.*, 2001) were grown at 30°C in LB medium. *Agrobacterium tumefaciens* NTL4 (pZLR4) (Cha *et al.*, 1998) was grown at 28°C in both Nutrient Agar (NA) plates and AB minimal medium (Chilton *et al.*, 1974). When required, antibiotics were added at the following concentrations: ampicillin (100 μg/ml), chloramphenicol (50 μg/ml), gentamicin (30 μg/ml), kanamycin (50 μg/ml), nalidixic acid (25 μg/ml), spectinomycin (100 μg/ml) and tetracycline (15 μg/ml). 5-Bromo-4-cloro-3-indoyl-β-D-galactopyranoside (X-gal) was used at 80 μg/ml when necessary.

PfvR proteins of *P. fuscovaginae* strain UPB0736 and LMG12428 were expressed in *E. coli* M15 as a 6-His-PfvR protein and expression plasmids were constructed as follows: *pfvR* genes were amplified by PCR using PFVR13F-PFVR13R and PFVR15F-PFVR15R primers for *pfvR* strain UPB0736 and *pfvR* strain LMG12428 respectively and cloned *Bam*HI-*Hin*dIII in pQE30 expression plasmid (Qiagen) generating pQER13 and pQER15 (Table 1).

4.5.2 Recombinant DNA techniques

DNA manipulations, including digestion with restriction enzymes, agarose gel electrophoresis, purification of DNA fragments, ligation with T4 ligase, DNA hybridization, radioactive labelling by random priming, and transformation of *E. coli*, were performed as described previously (Sambrook *et al.*, 1989). Southern hybridizations were performed by using N+Hybond membranes (Amersham Biosciences); plasmids were purified using Jet star columns (Genomed GmbH, Löhne, Germany) or by the alkaline lysis method (Birnboim, 1983); total DNA from *Pseudomonas* was isolated by Sarkosyl-pronase lysis as described previously (Better *et al.*, 1983). Triparental matings from *E. coli* to *P. fuscovaginae* were carried out with the helper strain *E. coli* (pRK2013) (Figurski and Helinski, 1979).

4.5.3 Cloning the AHL QS locus of *P. fuscovaginae* strains LMG12428 and UPB0736

A pLAFR3 cosmid library of partially digested *Eco*RI of *P. fuscovaginae* strain LMG12428 genomic DNA was constructed using Gigapack III XL-4 packaging kit as recommended by the supplier (Stratagene). The cosmid library of *P. fuscovaginae* strain LMG12428 harboured in *E. coli* was transferred by triparental conjugation to *C. violaceum* CV026 (Swift *et al.*, 1993). After overnight incubation at 30°C, the conjugations were plated on KB containing tetracycline for selection of transconjugants and ampicillin and spectinomycin for counter-selection of *E. coli*. These plates were incubated at 30°C for 24 h, transconjugants that turned purple were further assayed. One cosmid, named pSG100, could restore purple pigmentation in *C. violaceum* CV026. A 4 Kbp *Bam*HI fragment from pSG100 was subcloned in

pBluescriptKS, generating pSG100L plasmid. pSG100L could induce violacein production on solid KB medium in a streak plate assay (Piper *et al.*, 1993) using *C. violaceum* CV026. pSG100L was sequenced in both strands and shown to contain the AHL QS locus consisting of three genes, *pfvI-rsaL-pfvR* (Figure 2).

In order to clone the AHL QS system of *P. fuscovaginae* UPB0736, genomic DNA of strain UPB0736 was digested with *Hin*cII and ~5 Kbp fragments were cloned in pBluescriptKS since Southern analysis using the *pfvI* gene of strain LMG12428 as probe showed that the AHL QS system was present in a *Hin*cII fragment of approximately this size. The plasmid carrying the QS locus, pSG120, was then found via a colony blotting protocol (Amersham Biosciences) having a 4,7 Kbp insert which was consequently sequenced in both strands and confirmed to contain the AHL QS locus (Figure 2).

4.5.4 Construction of *P. fuscovaginae* UPB0736 - *pfvR* mutant

P. fuscovaginae UPB0736 *pfvR* 462 bp-internal fragment was amplified by PCR using pK13RF-pK13RR primers (Table 1) and cloned as an *Xba*I-*Xho*I fragment in pKNOCK-Km (Alexeyev, 1999) generating pKNR13. pKNR13 was then delivered by triparental conjugation to *P. fuscovaginae* UPB0736 as a suicide system in order to create *P. fuscovaginae* 13R (a *pfvR* knock-out mutant) via homologous recombination (Alexeyev, 1999). Selection was performed on KB medium containing ampicillin, chloramphenicol, nalidixic acid, spectinomycin and kanamycin. The marker exchange event in the mutant strain 13R was further confirmed by Southern blot analysis.

4.5.5 Reporter gene fusion assay

ß-galactosidase activities were determined during growth in LB medium essentially as described by Miller (Miller, 1972), with the modifications of Stachel et al. (Stachel *et al.*, 1985). All experiments were performed in triplicate. *E. coli* M15 (pQER13)(pMPI13) and *E. coli* M15 (pQER15)(pMPI15) were induced with IPTG (Isopropyl β-D-1-thiogalactopyranoside) at OD_{600} 0.6 and ß-galactosidase activities were determined one hour after induction.

4.5.6 Purification, detection and characterization of AHLs

Purification, detection, and characterization of AHLs were performed as previously described (McClean *et al.*, 1997). Briefly, *P. fuscovaginae* strains were grown overnight in M9 minimal medium supplemented with citric acid, casamino acids and 10% KB medium. 30 ml of supernatants of the cultures were extracted two times with the same volume of ethyl acetate-0.1% acetic acid, the extracts were then dried and resuspended in ethyl acetate. TLC analysis was performed using a C_{18} reverse-phase TLC plates in an elution buffer 60% (vol/vol) methanol-water. Synthetic AHLs were used as standard molecules (acquired from P. Williams, University of Nottingham, Nottingham, UK). Characterization of the AHLs was based on the evaluation of the Rf, shapes of the spots and on the differential responses of the AHL bacterial sensor strain (Steindler and Venturi, 2007). The AHL molecules on the TLC plate were detected by overlaying the TLC plate with a thin layer of AB top agar and X-gal seeded with *A. tumefaciens* NTL4 (pZLR4). T-streak plate assays (Piper *et al.*, 1993) were performed on solid KB medium using *C. violaceum* CV026 and F117 (pKRC12) AHLs biosensors.

4.5.7 Plant inoculations

The infection was performed on four weeks old *Chenopodium quinoa* plants, choosing the fifth, the sixth and the seventh leaves from the apex. *C. quinoa* was grown in a greenhouse at 28°C, humidity 70% with a photoperiod of 16 hrs light and 8 hrs dark. *P. fuscovaginae* was grown on KB-agar plates, bacteria were collected with a pin and the petiole was then pierced passing through all the tissues. The control experiment was performed by piercing the petiole with a clean sterile pin. Each treatment was done on six plants and three leaves per plant were inoculated. After the infection, the plants were sprinkled with water and placed into a humid chamber for four days, at 28°C, 100% humidity and with a photoperiod of 16 hours light and 8 hours dark. Infected plants were placed for one day outside of the humid chamber before the evaluation. After five days, disease index was performed on a 0-5 severity scale; 0: no symptoms, 1: necrosis on less than 2 mm around the puncture, 2: necrosis from 2 to 10 mm around the puncture, 3: necrosis from 2 to 10 mm around the puncture and bending of the petiole, 4: collapse of the petiole and 5: wilting of the leaf (Figure 5). The infection on rice (*Oryza sativa*, cv. IR24; provided by IRRI, Philippines) was performed on three weeks old plants grown in the greenhouse at 28°C, humidity 70% with a photoperiod of 16 hrs light and 8 hrs dark. The stem was pierced with a syringe full of water paying attention not to pass through the stem but maintaining the tip of the needle in the center of the shaft. The water was inoculated so as to watersoak all of the stem. *P. fuscovaginae* was grown on KB plates, bacteria were then collected from the plate with a pin which was used to pierce in the same point where the water was injected, passing the pin through the entire stem. The

control experiment was performed by injecting the water into the stem and, with the same needle, passing through the entire shaft. Each treatment was performed on 40 plants. After the infection, the plants were sprinkled with water and placed into a humid chamber for four days, at 28°C, 100% of humidity and with a photoperiod of 16 hours of light and 8 hours of dark. The evaluation was done after five days from the infection, the plants were placed for one day outside of the humid chamber before disease evaluation The disease index was on a 0-5 disease severity scale; 0: no symptoms, only the sign of the puncture, 1: necrosis around the puncture till 1 cm, 2: necrosis around the puncture and chlorosis from 1 to 3 cm on the stem, 3: necrosis around the puncture till 5 cm on the stem, 4: necrosis around the puncture for the two third of the new leaf and 5: necrosis around the puncture throughout the new leaf (Figure 6).

Statistical analyses were performed by means of SPSS 15.0 software. Score ratings in the two groups were analysed statistically in SPSS program using the Kruskal-Wallis multiple comparison test. Then the two groups has been tested by the Mann-Whitney comparison test. The minimum level of statistical significance was set at $p=0.05$.

HR was performed on 10 weeks old tobacco (*Nicotiana tabacum*) leaves by injection with a needle-less hypodermic syringe. Two-three sites on each leaf were infiltrated with a 10^8 CFU mL^{-1} bacterial suspension. As a control sterile culture medium was also injected into three sites on a tobacco leaf. Infected plants were placed at 26 °C in greenhouse with 70% humidity. The reaction at the infiltrated sites was scored 24, 48 and 72 hours after injection.

4.5.8 Exoenzyme production

Both proteolytic and lipolytic activity were determined on KB agar plates supplemented with 2% w/v powder skim milk and 1% v/v tributyrin respectively as previously described (Frazier and Rupp, 1928; Huber *et al.*, 2001; Knaysi, 1941). After inoculation, plates were incubated at 30 °C for three days.

4.5.9 Motility assays

The swimming assay was performed on 0.3% Difco agar KB plates (Burkart *et al.*, 1998), with antibiotic selection when necessary. Plates were dried for at least one hour under a sterile hood and the inoculation was performed from a culture at OD_{600} 2.0 using sterile toothpicks. Plates were incubated in humid conditions at 30 °C and the result was evaluated three days after inoculation.

The swarming assay was performed in M8 minimal medium used in this assay consisted of 20 mM Na_2HPO_4, 3 mM KH_2PO_4, 1 mM NaCl, 0.2% glucose, 0.05% glutamate, 0.5% Difco agar. M8 plates were dried for at least one hour under sterile hood and the inoculation was performed from a culture at OD_{600} 2.0 using sterilized toothpicks. Plates were incubated at 30 °C and the result was evaluated 7 days after inoculation.

4.5.10 DNA sequencing and nucleotide sequence accession numbers

All DNA sequencing was performed by Macrogen (www.macrogen.com) and the nucleotide sequences of the 2600 bp *Hin*cII fragment harboring *pfvI*, *rsaL* and *pfvR* of strain UPB0736 and the 2600 bp *Bam*HI fragment of strain LMG12428 harboring

pfvI, *rsaL* and *pfvR* have been deposited in GenBank/EMBL/DDBJ under the following accession numbers respectively AM943857 and AM943858.

4.5.11 Statistical analysis

P values were calculated using the ANOVA one way test led by MS excel. P values ≤0,05 were considered to be statistically significant.

4.6 ACKNOWLEDGEMENTS

S.F. is beneficiary of an ICGEB fellowship. L.C and M.M. and research activities at the Biosafety Outstation are supported by the Fondazione Cassamarca, Treviso, Italy. We wish to thank Francesca de Terlizzi for assistance in statistical analysis, Laura Chiappetta for the tobacco plants and Gabriele Giachin for his contribution in the project.

4.7 REFERENCES

Alexeyev, M.F. (1999) The pKNOCK series of broad-host-range mobilizable suicide vectors for gene knockout and targeted DNA insertion into the chromosome of gram-negative bacteria. *Biotechniques* **26**: 824-826, 828.

Ballio, A., Barra, D., Bossa, F., Collina, A., Grgurina, I., Marino, G., Moneti, G., Paci, M., Pucci, P., Segre, A., and et al. (1991) Syringopeptins, new phytotoxic lipodepsipeptides of *Pseudomonas syringae* pv. *syringae*. *FEBS Lett* **291**: 109-112.

Ballio, A., Bossa, F., Camoni, L., Di Giorgio, D., Flamand, M.-C., Maraite, H., Nitti, G., Pucci, P., and Scaloni, A. (1996) Structure of fuscopeptins, phytotoxic metabolites of *Pseudomonas fuscovaginae*. *FEBS Lett* **381**: 213-216.

Bassler, B.L. (1999) How bacteria talk to each other: regulation of gene expression by quorum sensing. *Curr Opin Microbiol* **2**: 582-587.

Batoko, H., de Kerchove d'Exaerde, A., Kinet, J.M., Bouharmont, J., Gage, R.A., Maraite, H., and Boutry, M. (1998) Modulation of plant plasma membrane H+-ATPase by phytotoxic lipodepsipeptides produced by the plant pathogen *Pseudomonas fuscovaginae*. *Biochim Biophys Acta* **1372**: 216-226.

Bertani, I., and Venturi, V. (2004) Regulation of the *N*-acyl homoserine lactone-dependent quorum-sensing system in rhizosphere *Pseudomonas putida* WCS358 and cross-talk with the stationary-phase RpoS sigma factor and the global regulator GacA. *Appl Environ Microbiol* **70**: 5493-5502.

Better, M., Lewis, B., Corbin, D., Ditta, G., and Helinski, D.R. (1983) Structural relationships among Rhizobium meliloti symbiotic promoters. *Cell* **35**: 479-485.

Birnboim, H.C. (1983) A rapid alkaline extraction method for the isolation of plasmid DNA. *Methods Enzymol* **100**: 243-255.

Burkart, M., Toguchi, A., and Harshey, R.M. (1998) The chemotaxis system, but not chemotaxis, is essential for swarming motility in *Escherichia coli*. *Proc Natl Acad Sci U S A* **95**: 2568-2573.

Cha, C., Gao, P., Chen, Y.C., Shaw, P.D., and Farrand, S.K. (1998) Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. *Mol Plant Microbe Interact* **11**: 1119-1129.

Chai, Y., and Winans, S.C. (2004) Site-directed mutagenesis of a LuxR-type quorum-sensing transcription factor: alteration of autoinducer specificity. *Mol Microbiol* **51**: 765-776.

Chilton, M.D., Currier, T.C., Farrand, S.K., Bendich, A.J., Gordon, M.P., and Nester, E.W. (1974) *Agrobacterium tumefaciens* DNA and PS8 bacteriophage DNA not detected in crown gall tumors. *Proc Natl Acad Sci U S A* **71**: 3672-3676.

Collins, C.H., Arnold, F.H., and Leadbetter, J.R. (2005) Directed evolution of *Vibrio fischeri* LuxR for increased sensitivity to a broad spectrum of acyl-homoserine lactones. *Mol Microbiol* **55**: 712-723.

de Kievit, T., Seed, P.C., Nezezon, J., Passador, L., and Iglewski, B.H. (1999) RsaL, a novel repressor of virulence gene expression in Pseudomonas aeruginosa. *J Bacteriol* **181**: 2175-2184.

Duveiller, E., Miyajima, K., Snacken, F., Autrique, A., and Maraite, H. (1988) Characterization of *Pseudomonas fuscovaginae* and differentiation from other fluorescent Pseudomonads occurring on rice in Burundi. *J. Phytopathology* **122**: 97-107.

Duveiller, E., Snacken, F., and Maraite, H. (1989) First detection of *Pseudomonas fuscovaginae* on maize and sorghum in Burundi. *Plant Disease* **73**: 514-517.

Duveiller, E. (1990) Bacterial sheath rot of wheat caused by *Pseudomonas fuscovaginae* in the highlands of Mexico. *Plant Disease* **74**: 932-935.

Figurski, D.H., and Helinski, D.R. (1979) Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. *Proc Natl Acad Sci USA* **76**: 1648-1652.

Flamand, M.-C., Pelsser, S., Ewbank, E., and Maraite, H. (1996) Production of syringotoxin and other bioactive peptides by *Pseudomonas fuscovaginae*. *Physiol Mol Plant Pathol* **48**: 217-231.

Frazier, W.C., and Rupp, P. (1928) Studies on the proteolytic bacteria of milk I. A Medium for the direct isolation of caseolytic milk bacteria. *J Bacteriol* **16**: 57-63.

Fuqua, C., Parsek, M.R., and Greenberg, E.P. (2001) Regulation of gene expression by cell-to-cell communication: acyl-homoserine lactone quorum sensing. *Annu Rev Genet* **35**: 439-468.

Fuqua, W.C., Winans, S.C., and Greenberg, E.P. (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators. *J Bacteriol* **176**: 269-275.

Gross, D.C., and Cody, Y.S. (1985) Mechanisms of plant pathogenesis by *Pseudomonas* species. *Can J Microbiol* **31**: 403-410.

Gross, D.C. (1991) Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. *Ann Rev Phytopathol* **29**: 247-278.

GuanLin, X. (2003) First report of sheath brown rot of rice in China and characterization of the causal organism by phenotypic tests and Biolog. *International Rice Research Notes* **28**: 50-52.

Harshey, R.M. (2003) Bacterial motility on a surface: many ways to a common goal. *Annu Rev Microbiol* **57**: 249-273.

Huber, B., Riedel, K., Hentzer, M., Heydorn, A., Gotschlich, A., Givskov, M., Molin, S., and Eberl, L. (2001) The cep quorum-sensing system of *Burkholderia cepacia* H111 controls biofilm formation and swarming motility. *Microbiology* **147**: 2517-2528.

Hutchison, M.L., Tester, M.A., and Gross, D.C. (1995) Role of Biosurfactant and Ion Channel-Forming Activities of Syringomycin Tranransmembrane Ion Flux: A Model for the Mechanism of Action in the Plant-Pathogen Interaction. *Mol Plant Microbe-Interact* **8**: 610-620.

Hutchison, M.L., and Gross, D.C. (1997) Lipopeptide Phytotoxins Produced by Pseudomonas syringae pv. syringae: Comparison of the Biosurfactant and Ion Channel-Forming Activities of Syringopeptin and Syringomycin. *Mol Plant Microbe-Interact* **10**: 347-354.

Kim, J., Kim, J.G., Kang, Y., Jang, J.Y., Jog, G.J., Lim, J.Y., Kim, S., Suga, H., Nagamatsu, T., and Hwang, I. (2004) Quorum sensing and the LysR-type transcriptional activator ToxR regulate toxoflavin biosynthesis and transport in *Burkholderia glumae*. *Mol Microbiol* **54**: 921-934.

King, E.O., Ward, M.K., and Raney, D.E. (1954) Two simple media for the demonstration of pyocyanin and fluorescin. *J Lab Clin Med* **44**: 301-307.

Knaysi, G. (1941) On the Use of Basic Dyes for the Demonstration of the Hydrolysis of Fat. *J Bacteriol* **42**: 587-589.

Kohler, T., Curty, L.K., Barja, F., van Delden, C., and Pechere, J.C. (2000) Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *J Bacteriol* **182**: 5990-5996.

Lazdunski, A.M., Ventre, I., and Sturgis, J.N. (2004) Regulatory circuits and communication in Gram-negative bacteria. *Nat Rev Microbiol* **2**: 581-592.

Magazin, M.D., Moores, J.C., and Leong, J. (1986) Cloning of the gene coding for the outer membrane receptor protein for ferric pseudobactin, a siderophore from a plant growth-promoting. *Pseudomonas* strain. *J Biol Chem* **261**: 795-799.

McClean, K.H., Winson, M.K., Fish, L., Taylor, A., Chhabra, S.R., Camara, M., Daykin, M., Lamb, J.H., Swift, S., Bycroft, B.W., Stewart, G.S., and Williams, P. (1997) Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. *Microbiology* **143 (Pt 12)**: 3703-3711.

Miller, J.H. (1972) Experiments in molecular genetics. *Cold Spring Harbor Laboratory Cold Spring Harbor , N.Y.*

Miyajima, K., Tanii, A., and Akita, T. (1983) *Pseudomonas fuscovaginae* sp. nvm. rev. *Int. J. Syst. Bacteriol.* **33**: 656-657.

Pearson, J.P., Passador, L., Iglewski, B.H., and Greenberg, E.P. (1995) A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **92**: 1490-1494.

Pesci, E.C., Pearson, J.P., Seed, P.C., and Iglewski, B.H. (1997) Regulation of *las* and *rhl* quorum sensing in *Pseudomonas aeruginosa*. *J Bacteriol* **179**: 3127-3132.

Piper, K.R., Beck von Bodman, S., and Farrand, S.K. (1993) Conjugation factor of *Agrobacterium tumefaciens* regulates Ti plasmid transfer by autoinduction. *Nature* **362**: 448-450.

Rampioni, G., Bertani, I., Zennaro, E., Polticelli, F., Venturi, V., and Leoni, L. (2006) The quorum-sensing negative regulator RsaL of *Pseudomonas aeruginosa* binds to the *lasI* promoter. *J Bacteriol* **188**: 815-819.

Riedel, K., Hentzer, M., Geisenberger, O., Huber, B., Steidle, A., Wu, H., Hoiby, N., Givskov, M., Molin, S., and Eberl, L. (2001) *N*-acylhomoserine-lactone-mediated communication between

Pseudomonas aeruginosa and *Burkholderia cepacia* in mixed biofilms. *Microbiology* **147**: 3249-3262.

Rott, P., and Notteghem, J.L. (1989) Identification and Characterization of *Pseudomonas fuscovaginae*, the Causal Agent of Bacterial Sheath Brown Rot, from Madagascar and Other Countries. *Plant Disease* **73**: 133-137.

Rott, P. (1991) Identification of *Pseudomonas fuscovaginae* with Biochemical, Serological, and Pathogenicity Tests. *Plant Disease* **75**: 843-846.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed. *Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.*

Schuster, M., Urbanowski, M.L., and Greenberg, E.P. (2004) Promoter specificity in *Pseudomonas aeruginosa* quorum sensing revealed by DNA binding of purified LasR. *Proc Natl Acad Sci U S A* **101**: 15833-15839.

Shaw, P.D., Ping, G., Daly, S.L., Cha, C., Cronan, J.E., Jr., Rinehart, K.L., and Farrand, S.K. (1997) Detecting and characterizing *N*-acyl-homoserine lactone signal molecules by thin-layer chromatography. *Proc Natl Acad Sci U S A* **94**: 6036-6041.

Spaink, H.P., Okker, R.J.H., Wijffelmann, C.A., Pees, E., and B.J.J., L. (1987) Promoter in the nodulation region of the *Rhizobium leguminosarum* Sym plasmid pRL1JI. *Plant Mol. Biol.* **9**: 27-39.

Stachel, S.E., An, G., Flores, C., and Nester, E.W. (1985) A Tn3 *lacZ* transposon for the random generation of beta-galactosidase gene fusions: application to the analysis of gene expression in *Agrobacterium*. *Embo J* **4**: 891-898.

Steindler, L., and Venturi, V. (2007) Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol Lett* **266**: 1-9.

Swift, S., Winson, M.K., Chan, P.F., Bainton, N.J., Birdsall, M., Reeves, P.J., Rees, C.E., Chhabra, S.R., Hill, P.J., Throup, J.P., and et al. (1993) A novel strategy for the isolation of *luxI* homologues: evidence for the widespread distribution of a LuxR:LuxI superfamily in enteric bacteria. *Mol Microbiol* **10**: 511-520.

Swift, S., Bainton, N.J., and Winson, M.K. (1994) Gram-negative bacterial communication by *N*-acyl homoserine lactones: a universal language? *Trends Microbiol* **2**: 193-198.

Tanii, A., Miyajima, K., and Akita, T. (1976) The sheath brown rot disease of rice and its causal bacterium *Pseudomonas fuscovaginae* sp. nov. *Ann. Phytopathol. Soc. Jpn.* **42**: 540-548.

Urbanowski, M.L., Lostroh, C.P., and Greenberg, E.P. (2004) Reversible acyl-homoserine lactone binding to purified *Vibrio fischeri* LuxR protein. *J Bacteriol* **186**: 631-637.

Venturi, V. (2006) Regulation of quorum sensing in *Pseudomonas*. *FEMS Microbiol Rev* **30**: 274-291.

Von Bodman, S.B., Bauer, W.D., and Coplin, D.L. (2003) Quorum sensing in plant-pathogenic bacteria. *Annu Rev Phytopathol* **41**: 455-482.

Whitehead, N.A., Barnard, A.M., Slater, H., Simpson, N.J., and Salmond, G.P. (2001) Quorum-sensing in Gram-negative bacteria. *FEMS Microbiol Rev* **25**: 365-404.

Winson, M.K., Swift, S., Fish, L., Throup, J.P., Jorgensen, F., Chhabra, S.R., Bycroft, B.W., Williams, P., and Stewart, G.S. (1998) Construction and analysis of *luxCDABE*-based plasmid sensor for investigatig *N*-acyl homoserine lactone-mediated quorum sensing. *FEMS Microbiol Lett* **163**: 185-192.

Zeigler, R.S., and Alvarez, E. (1987) Bacterial Sheath Brown Rot of Rice by *Pseudomonas fuscovaginae* in Latin America. *Plant Disease* **71**: 592-597.

Zeigler, R.S. (1990) Characteristics of *Pseudomonas* spp. Causing Grain Discoloration and Sheath Rot of Rice, and Associated Pseudomonad Epiphytes. *Plant Disease* **74**: 917-922.

Zhang, L.H., and Dong, Y.H. (2004) Quorum sensing and signal interference: diverse implications. *Mol Microbiol* **53**: 1563-1571.

Zhu, J., and Winans, S.C. (2001) The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization. *Proc Natl Acad Sci U S A* **98**: 1507-1512.

SUMMARISING DISCUSSION

5.1 SUMMARISING DISCUSSION

The aim of these studies was to investigate the quorum sensing (QS) systems based on *N*-acyl homoserine lactone (AHL) signal molecules in two important rice pathogens, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) and *Pseudomonas fuscovaginae*. When these investigations began almost four years ago, there were no reports of AHL communication in these two rice-associated bacterial species. Our studies revealed two very different situations: (i) *Xoo* did not present the typical AHL quorum sensing (QS) system for intra/inter-species communication. However it was able to detect a plant signal molecule establishing an inter-kingdom communication via a LuxR-family regulator closely related to AHL QS and (ii) in *P. fuscovaginae* we found a typical AHL QS system, highly conserved and highly similar to the QS systems present in *Pseudomonas aeruginosa* and *Pseudomonas putida* species. Due to these differences the following summarizing discussion will be divided into two parts. Since the three chapters in this thesis which present research data already contain a discussion, in this section a summary of the results is outlined and discussed in a more general context.

5.1.1 Xanthomonas oryzae pv. *oryzae*

Xanthomonas oryzae pv. *oryzae* (*Xoo*) is the causal agent of bacterial leaf blight of rice (*Oryza sativa*). It is a vascular disease eventually clogging xylem vessels causing wilting, if infection occurs in earlier growth stages, or lesions of leaf blight if it occurs in later stages. The main objective of the project was the characterization of the AHL QS system and regulation in *Xoo*. The most common quorum sensing system in

Gram-negative bacteria employs *N*-acyl homoserine lactone (AHL) as signal molecule. AHLs allow bacteria to monitor their cell density and it is commonly used to synchronize/coordinate the expression of virulence associated factors. What was established fairly quickly using AHL bacterial biosensors is that *Xoo* does not produce AHLs. This was then confirmed once the first *Xoo* genome was published in January 2005 (Lee *et al.*, 2005) which did not reveal the presence in the genome of a *luxI*-family gene, essential for AHLs synthesis. However an unpaired or orphan *luxR*-homolog gene closely related to QS *luxR* family genes was found in the *Xoo* genome. Interestingly this *Xoo* LuxR-family protein, which was designated OryR, displayed the classical structure of the AHL QS LuxR-family regulators: it possessed an AHL-binding domain and a helix-turn-helix DNA binding motif, leading to the hypothesis of a possible OryR-AHL interaction during its regulatory activity. Such a scenario was previously reported in *Escherichia coli* and *Salmonella enterica*, where the protein SdiA enables these bacteria, which do not synthesize AHLs, to detect and respond to exogenous AHLs produced by other bacterial species (Ahmer, 2004). To test this hypothesis we analysed OryR solubility in the presence of many different AHLs. It was previously demonstrated for LuxR in *Vibrio fischeri*, for LasR in *Pseudomonas aeruginosa* and for TraR in *Agrobacterium tumefaciens* that LuxR-family quorum sensing proteins, when over-expressed, are highly insoluble, however in the presence of their cognate AHL molecule, they become soluble (Schuster *et al.*, 2004; Urbanowski *et al.*, 2004; Vannini *et al.*, 2002). OryR was over-expressed in *E. coli* in the presence of different AHLs and an affinity chromatography was performed

after an extraction under native conditions to recover a soluble form of the protein. No solubility has been observed with any of the AHLs tested and it was therefore concluded that OryR, unlike SdiA, was not binding AHLs. Since *Xoo* is a rice pathogen and its aim is rice xylem vessel colonization, it was hypothesized that a possible regulatory function of OryR was during the infection stages, thus possibly interacting with a plant molecule. To verify this possibility we created a new culture medium containing macerated rice and analysed OryR solubility under these conditions. Interestingly some soluble OryR in the elution fraction was detected, indicating possibly that something in macerated rice can bind and solubilize OryR.

In order to reproduce the host environment, we also tested whether xylem sap, recovered from infected leaves, could solubilize OryR. Xylem sap had been collected in different periods from 3 to 14 days after *Xoo* infection and OryR solubilization was tested by over-expressing the protein in *E. coli* in the presence of xylem sap in the medium. Western blot analysis revealed highest amounts of the soluble form of OryR in the presence of xylem sap collected 10 days after infection. From this result we can assume that probably the amount of rice signal molecule (RSM) increased during infection, reaching a peak after 10 days from the infection. A low amount of the soluble form of OryR detected in the presence of non-infected rice xylem sap indicated that the unknown RSM was produced by the rice plant. It is therefore possible that the RSM is a low molecular weight compound (possibly a secondary metabolite) which the plant produces more under pathogen attack (Dixon, 2001).

OryR was found to be highly conserved among *Xoo* strains, as demonstrated from the Southern and Western blot analysis. OryR orthologues are also present in *X.*

campestris pv. *campestris* and *X. axonopodis* pv. *citri*, indicating a common ancestral origin and a possible important function of OryR in *Xanthomonas* spp. as this gene has been conserved during evolution.

To investigate the involvement of OryR in *Xoo* rice virulence, three *Xoo* strains were mutagenized, creating three *Xoo oryR* knock-out mutants. From the pathogenicity profile of the *oryR* mutants vs the parental strains it was clear that OryR was involved in *Xoo* virulence, as demonstrated by the reduction of leaf lesion length for all the three strains tested. The reduction in pathogenicity was however clearest for the most pathogenic strain, especially when the bacterial inoculum was reduced, but was less evident for the other two strains of which the parent was less pathogenic on rice. OryR is therefore certainly involved in *Xoo* rice pathogenicity, probably *via* the regulation of virulence gene(s).

In order to test our OryR working model, we studied OryR target promoters in the presence of macerated rice, as we hypothesized that OryR needs RSM to carry out its regulatory function. In *Xanthomonas campestris* pv. *campestris* (*Xcc*), which is closely related to *Xoo*, it was reported that an OryR ortholog, termed XccR, had regulatory function on the downstream adjacent gene encoding for the proline iminopeptidase (Pip) (Zhang *et al.*, 2007). It was demonstrated that Pip is a periplasmic protein involved in virulence (its mode of action is currently unknown), directly regulated by XccR in *Xcc* in the host plant in response to an unknown plant compound (Zhang *et al.*, 2007). We analysed *pip* promoter regulation via OryR in *Xoo* by firstly creating a new promoter probe plasmid, designated pSS122; the *pip* promoter was then cloned upstream of the promoterless *uidA* gene, encoding β-

glucuronidase. *Pip* promoter activity was measured after growing *Xoo* in different culture media, in the presence and in the absence of macerated rice. Our results demonstrated that OryR is necessary for *pip* promoter activation, since no promoter activity has been observed in *Xoo* OryR mutants. It was demonstrated for XccR in *Xcc* that the protein binds a canonical QS *lux*-box present in the *pip* promoter region (Zhang *et al.*, 2007); a highly conserved QS *lux*-box was also found in the *pip* promoter region of *Xoo* leading to the hypothesis that this *lux*-box was the possible OryR binding site during *pip* promoter regulation. It is therefore tempting to conclude that OryR in *Xoo* and XccR in *Xcc* function in the same manner, but only when the bacterium is colonizing the host plant. It was of interest to determine whether OryR was able to regulate its own activity, so we tested the *oryR* promoter activity using the pSS122 reporter plasmid, in the presence and in the absence or rice in the culture medium. Surprisingly we observed that OryR could regulate its own promoter in a negative manner, independently of the RSM. We identified a weak QS *lux*-box sequence in the *oryR* promoter region. However, due to the very low consensus, we cannot conclude or convincingly speculate that OryR could bind this sequence. The putative *oryR lux*-box is imperfect, as one of the two highly conserved regions was not perfectly conserved, and this could possibly explain the regulation of OryR on its promoter even when not bound to the signal molecule; further experimentation is necessary in order to test this possibility. It was established that the OryR protein was present in *Xoo* when grown in minimal defined medium only in the presence of macerated rice but not in a minimal medium alone. This result was further confirmed testing *oryR* promoter activity on growing *Xoo* in a minimal medium with and without

macerated rice. OryR was expressed at a basal level when *Xoo* was grown in the minimum medium, whereas the expression was almost three times higher when rice was added to the medium, indicating again that *oryR* was itself regulated by an unknown regulator being induced *in planta*.

Analysing the secreted protein profile of *Xoo*, grown in the presence and in the absence of rice, it was observed that an approximately 60 KDa protein was present only when *Xoo* was grown with rice in the culture medium. This protein was however not detected in the *Xoo oryR* mutant. This protein was identified as a 1,4-β-cellobiosidase (CbsA), important for the hydrolysis of 1,4-β-D-glucosidic linkages in cellulose. CbsA was identified as one of the *Xoo* secreted proteins involved in virulence as the ability to cause lesions in rice of a *Xoo cbs* mutant was reduced (Jha *et al.*, 2007). To further confirm possible *cbsA* regulation via OryR, we cloned the *cbsA* promoter in the pSS122 plasmid and tested the promoter activity in *Xoo* grown in the presence and in the absence of macerated rice. Surprisingly our results indicated a reduction in *cbsA* expression in the presence of rice. However its activity was OryR dependent under all conditions tested and it was independent of the RSM. Since CbsA was detected in highest amounts when *Xoo* was grown in the presence of macerated rice, we cannot exclude a possible subsequent post-transcriptional regulation. As *cbsA* expression was tightly dependent on OryR, at this stage we cannot state whether OryR regulation on the *cbsA* promoter is direct or indirect. In the *cbsA* promoter a weak *lux*-box sequence is present but, as for the *oryR* promoter, this sequence was imperfect. From this evidence we can speculate that possibly the four nucleotides at the beginning of the sequence are critical for OryR binding (these sequences are present

in all putative *lux*-boxes, ie. in *pip*, *oryR*, and *cbsA* promoter) while the last three nucleotides of the putative *cbsA* and *oryR lux*-boxes could be important for the specificity of OryR bound to the RSM.

Xcc produces the QS signal molecule designated DSF (diffusible signal molecule), involved in the regulation of biofilm dispersal and production of virulence factors (Barber *et al.*, 1997; Dow *et al.*, 2003). DSF production and regulation is under the control of the *rpfA-H* cluster, in particular the gene *rpfF* is responsible for DSF synthesis (Barber *et al.*, 1997). It was of interest to determine whether OryR was regulating DSF production since the DSF system is also found in *Xoo* (Chatterjee and Sonti, 2002). DSF was extracted from the *Xoo* parental and *Xoo oryR* mutant, grown both in the presence and in the absence of rice, and differences in DSF production were evaluated using a *Xcc* DSF biosensor strain. Our results demonstrated that almost the same amount of DSF was extracted from *Xoo* parental and *Xoo* OryR mutant, both in the presence and in the absence of rice. To further support this result, the *rpfF* promoter was tested for OryR regulation, in the presence and in the absence of rice, confirming that OryR was not involved in *rpfF* regulation. It was concluded that OryR was probably not connected with the DSF system. At this stage we cannot exclude a possible regulation of the DSF QS system on *oryR*/OryR. A DSF genome scale analysis however did not detect that the DFS QS system was regulating *oryR* (He *et al.*, 2006).

From previous studies on OryR solubility we demonstrated that the rice signal molecule (RSM) recognized by OryR was not an *N*-acyl-homoserine lactone type molecule. We further confirmed this result by testing *pip* promoter activity in the

presence of many different AHLs. Our results showed that no *pip* promoter activity was induced in the presence of AHLs. The same experiment was performed in the presence of both the AHLs and the RSM, to demonstrate a possible inhibitory activity of AHLs on OryR due to a possible competition for binding. As expected we did not observe an inhibition of *pip* promoter activity consistent with the contention that OryR could not bind AHLs.

As RSM was expected to be a small molecule, media containing the RSM were fractionated in line with molecular size by progressive filtrations. Each fraction was tested on *pip* promoter induction, as *pip* expression was tightly regulated by OryR bound to the RSM. A strong *pip* promoter activity was detected only adding the <1KDa fraction to the growth medium, clearly indicating that the RSM is a small molecule. Our results demonstrated that RSM did not compete with AHLs for OryR binding, but we cannot however exclude the possibility that RSM could bind other LuxR-homolog regulators. Previous studies have shown that plants contain molecules able to activate bacterial AHL QS systems (Bauer and Mathesius, 2004). We therefore tested the possible binding between RSM to TraR of *A. tumefaciens*, (Cha *et al.*, 1998). The *A. tumefaciens* NT1(pZLQR) AHL biosensor strain was grown in the presence of the <1KDa fraction containing the RSM; a very slight increase of β-galactosidase activity was observed. It has been reported that rice contains AHL QS agonist molecules able to activate several AHL biosensors (Degrassi *et al.*, 2007), and we cannot exclude the presence in very low concentrations of these molecules in the fraction analysed. We can therefore conclude that RSM did not act as an AHL mimic, at least for the TraR regulator. Due to the very large number of low molecular weight

compounds produced by plants (Dixon, 2001), many of which produced in very low quantities, it will be a major task identifying the RSM interacting with OryR. One possibility could be a chemical extraction from the rice tissue, using a solvent (e.g. chloroform, ethyl acetate, ether...) in which the molecule results to be more soluble than in water. After a solvent extraction it would be possible to isolate the molecule using the HPLC chromatography; each fraction could then be tested by a bioassay, analysing for example the β-glucuronidase activity in *Xoo* XKK.12 (pPIP122) cells.

5.1.2 Pseudomonas fuscovaginae

Pseudomonas fuscovaginae is a Gram negative fluorescent pseudomonad pathogenic to rice (*Oryza sativa*) (Miyajima *et al.*, 1983; Tanii *et al.*, 1976). This opportunistic pathogen has been isolated in many countries and from different host plants, especially in tropical upland fields (Duveiller *et al.*, 1989), due to its ability to grow and colonize at high humidity and low temperatures (Miyajima *et al.*, 1983). *P. fuscovaginae* causes bacterial brown sheath rot on many cereals (Duveiller *et al.*, 1989; Duveiller, 1990) producing several phytotoxins (Gross and Cody, 1985; Gross, 1991).

The aim of this study was the characterization of the quorum sensing system (QS) in this emerging rice pathogen, as no molecular studies have been performed so far on this topic in this organism.

A collection of 15 *P. fuscovaginae* strains, isolated from diseased rice all over the world, were initially tested for AHL production. This screening was performed using many different AHL biosensors in order to detect the structurally different AHLs

(Steindler and Venturi, 2007). The results obtained, in particular using *A. tumefaciens* NT1(pZLR4) as biosensor, indicated a clear AHL production profile in three strains, LMG5742, UPB0898, and LMG12428, possibly identified as C_6-3oxo-AHL, C_8-3oxo-AHL, C_{10}-3oxo-AHL, C_{12}-3oxo-AHL. The remaining strains displayed a quite conserved AHL profile, producing mainly C_8-3oxo-AHL and C_{10}-3oxo-AHL. As AHLs were extracted from a large volume of culture supernatant in order to obtain detection, *P. fuscovaginae* probably produces low amounts of AHLs, indicating that the QS system could be regulated or possibly the system requires low concentrations of AHLs for its functioning.

To identify the QS genes we focused on two strains, UPB0736, that causes brown sheath rot on rice, and strain LMG12428, that causes leaf stripe on rice. We chose these strains not only for the differences in the lesion created on the rice plant, but also for the differences in the amount of AHLs produced. Firstly we constructed a cosmid library of strain LMG12428 and screened it by complementation in *trans* in the CV026 AHL biosensor. The cosmid containing the QS genes was isolated, subcloned and sequenced, leading to the identification of the typical *luxI-luxR* family genes. The AHL-QS system of *P. fuscovaginae*, designated as *pfvI/R*, was found to be highly similar to the PpuI/R and LasI/R systems of *P. putida* and *P. aeruginosa* respectively. In between the *pfvI/R* genes, the negative regulator *rsaL* was found which in *P. putida* and *P. aeruginosa* is an important negative regulator of the AHL synthase gene (de Kievit *et al.*, 1999; Rampioni *et al.*, 2006). A structurally similar *pfvI/R* QS locus was also then found in *P. fuscovaginae* UPB0736.

Due to the presence of the RsaL negative regulator, we can hypothesize that *pfvI* in strain UPB0736 is repressed by RsaL explaining the low amounts of AHLs detected in the TLC analysis. Similarly we can also speculate that in strain LMG12428, maybe RsaL is not repressing *pfvI* very strongly hence much more AHLs are produced. The *pfvI/R* QS system in *P. fuscovaginae* was found to be highly conserved among the 15 strains isolated from all over the world most likely revealing that the system has been conserved during evolution and that it could be part of the core genome of this species.

To better characterize PfvR AHL specificity, the protein was over-expressed in *E .coli* in the presence of different AHLs and cognate *pfvI* promoter activity was measured. For both strains, LMG12428 and UPB0736, *pfvI* promoter activity was induced in the presence of long chain AHLs, especially C_{10}-3oxo-AHL and C_{12}-3oxo-AHL; PfvR could also respond reasonably well to C_8-3oxo-AHL, C_{12}-AHL, C_{10}-AHL, C_{12}-3OH-AHL and C_{10}-3OH-AHL. This result was surprising since PfvR displayed rather relaxed specificity responding well to a rather large number of AHLs. In contrast, the similar systems found in *P. putida* and *P. aeruginosa* respond specifically only to C_{12}-3oxo-AHL. The reason for this relaxed specificity by PfvR is unknown. It could allow *P. fuscovaginae* to respond to more of the AHL it produces or possibly to exogenous ones produced by other bacterial species. Since we observed also short chain AHLs in the TLC profile, we cannot exclude the presence of a second QS system in *P. fuscovaginae*, as is the case in *P. aeruginosa* (Pearson *et al.*, 1995).

To evaluate the involvement of the QS system in pathogenicity and in disease development, we created a *pfvR* knock-out mutant in the very virulent *P. fuscovaginae*

strain UBB0736. Virulence of *P. fuscovaginae* UBB0736 parental strain and *P. fuscovaginae pfvR* mutant were evaluated on *Chenopodium quinoa* and *Oryza sativa* host plants by the stem puncture method. The *P. fuscovaginae pfvR* mutant was less aggressive compared to the parental strain for both plants tested, confirming an involvement of the QS system in virulence possibly by regulating virulence associated factors, for example the fuscopeptin phytotoxins that are known to be produced by this species (Ballio *et al.*, 1996).

It was previously reported that *P. fuscovaginae* is able to elicit a plant defence response in non-host plants like tobacco, causing an hypersensitive response (HR) (Duveiller *et al.*, 1988). To verify whether HR response was regulated by the QS system, we performed an HR assay of *P. fuscovaginae* UPB0736 parental strain compared to *P. fuscovaginae pfvR* mutant, by infiltrating tobacco leaf mesophyll with the bacterial suspensions. Interestingly the *pfvR* mutant was no longer able to cause an HR reaction on tobacco leaves, indicating an involvement of QS in the regulation of this process. It must be noted however that unusual necrotic symptoms were observed on tobacco leaves; these could be due to phytotoxins production rather than an hypersensitive response by the plant. This merits further attention before more concrete conclusions are drawn from this experiment.

Finally we tested swimming and swarming motility of the *P. fuscovaginae* UPB0736 parental strain and the *P. fuscovaginae pfvR* mutant, in laboratory conditions, in order to verify a possible involvement of QS in bacterial motility regulation. The *P. fuscovaginae* UPB0736 parental strain was able to swim on rich media plates. However the *P. fuscovaginae pfvR* mutant swam considerably faster compared to the

parental strain, creating numerous dendrites. Under the conditions we tested *P. fuscovaginae* UPB0736 was not able to undergo swarming motility, but interestingly the swimming profile observed in the *pfvR* mutant was very similar to the swarming motility of *P. aeruginosa* (Caiazza *et al.*, 2005). AHL QS is likely involved in the regulation of expression of many other genes; unfortunately, the lack of genome sequence data will render future investigations on the AHL QS regulon more laborious and time consuming.

5.2 REFERENCES

Ahmer, B.M. (2004) Cell-to-cell signalling in *Escherichia coli* and *Salmonella enterica*. *Mol Microbiol* **52**: 933-945.

Ballio, A., Bossa, F., Camoni, L., Di Giorgio, D., Flamand, M.-C., Maraite, H., Nitti, G., Pucci, P., and Scaloni, A. (1996) Structure of fuscopeptins, phytotoxic metabolites of *Pseudomonas fuscovaginae*. *FEBS Lett* **381**: 213-216.

Barber, C.E., Tang, J.L., Feng, J.X., Pan, M.Q., Wilson, T.J., Slater, H., Dow, J.M., Williams, P., and Daniels, M.J. (1997) A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by a small diffusible signal molecule. *Mol Microbiol* **24**: 555-566.

Bauer, W.D., and Mathesius, U. (2004) Plant responses to bacterial quorum sensing signals. *Curr Opin Plant Biol* **7**: 429-433.

Caiazza, N.C., Shanks, R.M., and O'Toole, G.A. (2005) Rhamnolipids modulate swarming motility patterns of *Pseudomonas aeruginosa*. *J Bacteriol* **187**: 7351-7361.

Cha, C., Gao, P., Chen, Y.C., Shaw, P.D., and Farrand, S.K. (1998) Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. *Mol Plant Microbe Interact* **11**: 1119-1129.

Chatterjee, S., and Sonti, R.V. (2002) *rpfF* mutants of *Xanthomonas oryzae* pv. *oryzae* are deficient for virulence and growth under low iron conditions. *Mol Plant Microbe Interact* **15**: 463-471.

de Kievit, T., Seed, P.C., Nezezon, J., Passador, L., and Iglewski, B.H. (1999) RsaL, a novel repressor of virulence gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* **181**: 2175-2184.

Degrassi, G., Devescovi, G., Solis, R., Steindler, L., and Venturi, V. (2007) *Oryza sativa* rice plants contain molecules that activate different quorum-sensing *N*-acyl homoserine lactone biosensors and are sensitive to the specific AiiA lactonase. *FEMS Microbiol Lett* **269**: 213-220.

Dixon, R.A. (2001) Natural products and plant disease resistance. *Nature* **411**: 843-847.

Dow, J.M., Crossman, L., Findlay, K., He, Y.Q., Feng, J.X., and Tang, J.L. (2003) Biofilm dispersal in *Xanthomonas campestris* is controlled by cell-cell signaling and is required for full virulence to plants. *Proc Natl Acad Sci U S A* **100**: 10995-11000.

Duveiller, E., Miyajima, K., Snacken, F., Autrique, A., and Maraite, H. (1988) Characterization of *Pseudomonas fuscovaginae* and differentiation from other fluorescent *Pseudomonads* occurring on rice in Burundi. *J. Phytopathology* **122**: 97-107.

Duveiller, E., Snacken, F., and Maraite, H. (1989) First detection of *Pseudomonas fuscovaginae* on maize and sorghum in Burundi. *Plant Disease* **73**: 514-517.

Duveiller, E. (1990) Bacterial sheath rot of wheat caused by *Pseudomonas fuscovaginae* in the highlands of Mexico. *Plant Disease* **74**: 932-935.

Gross, D.C., and Cody, Y.S. (1985) Mechanisms of plant pathogenesis by *Pseudomonas* species. *Canadian Journal of Microbiology* **31**: 403-410.

Gross, D.C. (1991) Molecular and genetic analysis of toxin production by pathovars of *Pseudomonas syringae*. *Annual Review of Phytopathology* **29**: 247-278.

He, Y.W., Wang, C., Zhou, L., Song, H., Dow, J.M., and Zhang, L.H. (2006) Dual signaling functions of the hybrid sensor kinase RpfC of *Xanthomonas campestris* involve either phosphorelay or receiver domain-protein interaction. *J Biol Chem* **281**: 33414-33421.

Jha, G., Rajeshwari, R., and Sonti, R.V. (2007) Functional interplay between two *Xanthomonas oryzae* pv. *oryzae* secretion systems in modulating virulence on rice. *Mol Plant Microbe Interact* **20**: 31-40.

Lee, B.M., Park, Y.J., Park, D.S., Kang, H.W., Kim, J.G., Song, E.S., Park, I.C., Yoon, U.H., Hahn, J.H., Koo, B.S., Lee, G.B., Kim, H., Park, H.S., Yoon, K.O., Kim, J.H., Jung, C.H., Koh, N.H., Seo, J.S., and Go, S.J. (2005) The genome sequence of *Xanthomonas oryzae* pathovar *oryzae* KACC10331, the bacterial blight pathogen of rice. *Nucleic Acids Res* **33**: 577-586.

Miyajima, K., Tanii, A., and Akita, T. (1983) *Pseudomonas fuscovaginae* sp. nvm. rev. *Int. J. Syst. Bacteriol.* **33**: 656-657.

Pearson, J.P., Passador, L., Iglewski, B.H., and Greenberg, E.P. (1995) A second *N*-acylhomoserine lactone signal produced by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **92**: 1490-1494.

Rampioni, G., Bertani, I., Zennaro, E., Polticelli, F., Venturi, V., and Leoni, L. (2006) The quorum-sensing negative regulator RsaL of *Pseudomonas aeruginosa* binds to the *lasI* promoter. *J Bacteriol* **188**: 815-819.

Schuster, M., Urbanowski, M.L., and Greenberg, E.P. (2004) Promoter specificity in *Pseudomonas aeruginosa* quorum sensing revealed by DNA binding of purified LasR. *Proc Natl Acad Sci U S A* **101**: 15833-15839.

Steindler, L., and Venturi, V. (2007) Detection of quorum-sensing *N*-acyl homoserine lactone signal molecules by bacterial biosensors. *FEMS Microbiol Lett* **266**: 1-9.

Tanii, A., Miyajima, K., and Akita, T. (1976) The sheath brown rot disease of rice and its causal bacterium *Pseudomonas fuscovaginae* sp. nov. *Ann. Phytopathol. Soc. Jpn.* **42**: 540-548.

Urbanowski, M.L., Lostroh, C.P., and Greenberg, E.P. (2004) Reversible acyl-homoserine lactone binding to purified *Vibrio fischeri* LuxR protein. *J Bacteriol* **186**: 631-637.

Vannini, A., Volpari, C., Gargioli, C., Muraglia, E., Cortese, R., De Francesco, R., Neddermann, P., and Marco, S.D. (2002) The crystal structure of the quorum sensing protein TraR bound to its autoinducer and target DNA. *Embo J* **21**: 4393-4401.

Zhang, L., Jia, Y., Wang, L., and Fang, R. (2007) A proline iminopeptidase gene upregulated *in planta* by a LuxR homologue is essential for pathogenicity of *Xanthomonas campestris* pv. *campestris*. *Mol Microbiol* **65**: 121-136.

APPENDIX

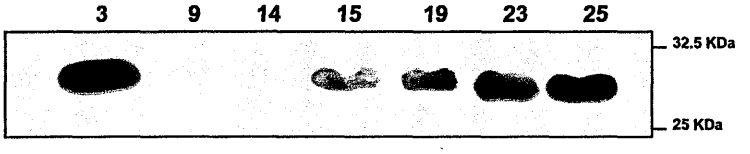


Figure 1: Western blot of the total protein extracts from the 7 strains which did not give a positive result in a Southern analysis using the complete *oryR* gene as probe. A protein of the expected size, recognized by the anti-OryR antibody, was detected in 5 of the 7 strains tested; this most likely is the OryR-homolo/ortholog. The numbers correspond to the following *Xoo* strains (see Table 1, chapter 2): 3- XAPC.10, 9-XAPC.20, 14-XKK.16, 15-XKPt.4, 19-XKV.5, 23-XTNP.4, 25-LMG5047.

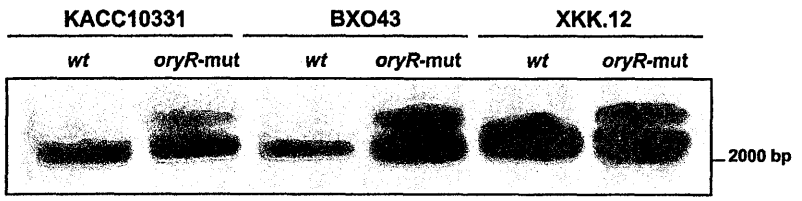


Figure 2: Southern blot analysis performed on the three *Xoo* strains, KACC10331, BXO43 and XKK.12, *versus* their corresponding *oryR* knock-out mutants. Chromosomal DNA was digested with *Nco*I restriction enzyme. The suicide vector pKNORY, used for the mutagenesis, contains a *NcoI* restriction site in the sequence thus generating, after digestion in the *oryR* mutant strains, two fragments of approximately 2100 and 2800 bp.

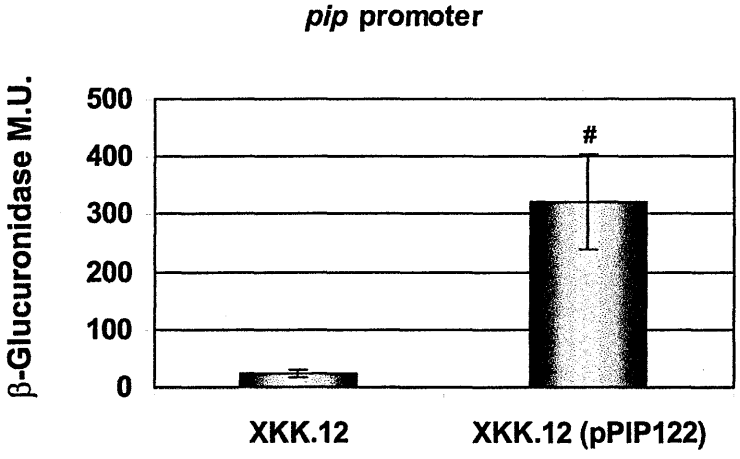


Figure 3: *pip* gene promoter activity *in planta*. β-glucuronidase assay was performed on *Xoo* XKK.12 (pPIP122) cells recovered from infected plants seven days after infection. Significant β-glucuronidase activity was detected despite that most of the bacterial cells recovered had lost the promoter-probe plasmid. The results are expressed as means ± STDEV, n=3. #, p< 0,003 compared to XKK.12 without the promoter-probe plasmid.

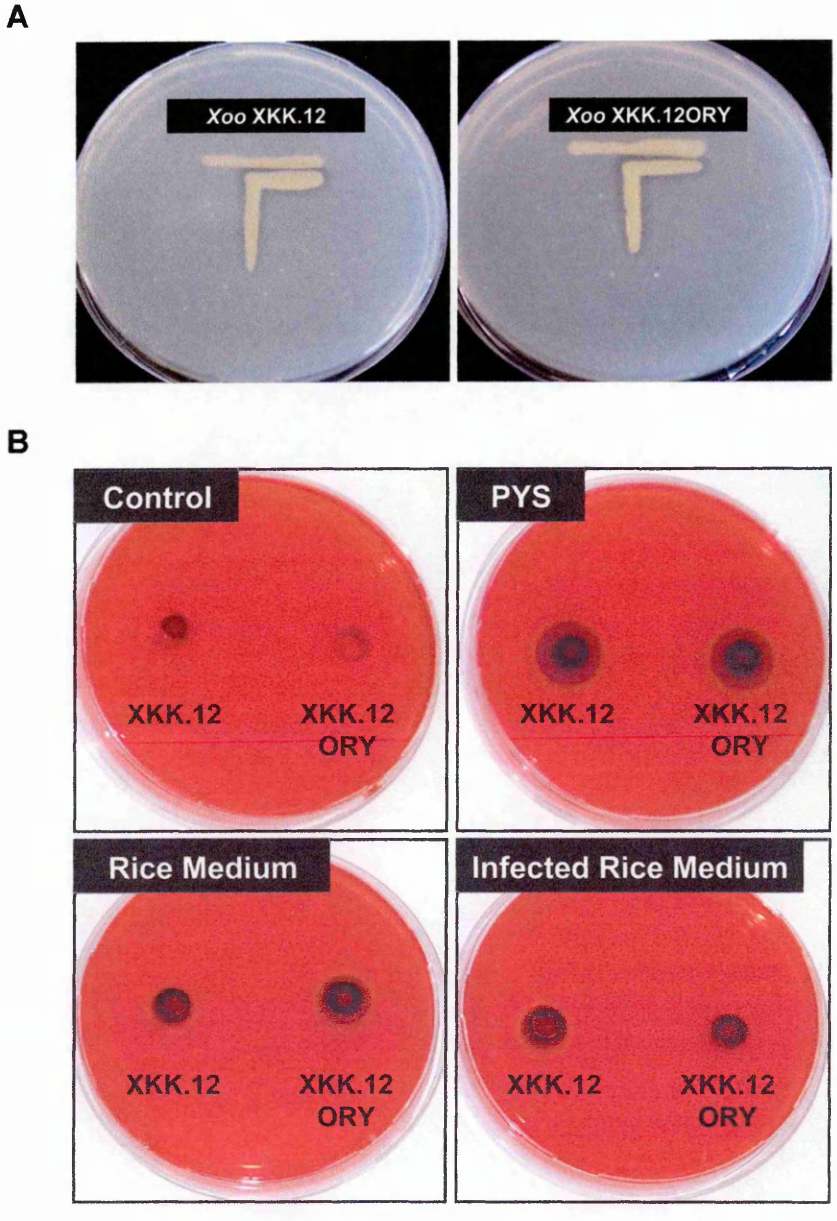


Figure 4: A: Plate test for DSF production. A plate containing skimmed milk (to indicate protease production) was inoculated with a streak of DSF producing *Xoo* and the DSF indicator strain *Xcc* 8523 was inoculated as an L-shaped streak. No differences were observed in protease activity induced by *Xoo* XKK.12 compared to protease activity induced by *Xoo* XKK.12ORY. B: CMC agar plate test for DSF production. DSF was extracted from both *Xoo* XKK.12 parental strain and *Xoo* XKK.12ORY, OryR mutant, grown in PYS medium, rice medium and infected rice medium; PYS alone was used as control. Extracted DSF was loaded into plate wells. Endoglucanase activity was detected as a clear halo around the wells. No differences were observed in the halo size induced by DSF extracted from *Xoo* XKK.12 and *Xoo* XKK.12ORY, both in the presence and in the absence of rice in the culture medium.

ANNEX

1. The *Pseudomonas fuscovagine* LMG 12428 quorum sensing locus (Chapter 4):

LOCUS AM943858 2600 bp DNA linear BCT 09-APR-2008
DEFINITION *Pseudomonas fuscovaginae* quorum sensing locus, strain LMG12428.
ACCESSION AM943858
VERSION AM943858.1 GI:182375405
KEYWORDS .
SOURCE *Pseudomonas fuscovaginae*
ORGANISM *Pseudomonas fuscovaginae*
Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;
Pseudomonadaceae; Pseudomonas.
REFERENCE 1
AUTHORS Ferluga,S., Cabrio,L., Mattiuzzo,M., Maraite,H. and Venturi,V.
TITLE The plant opportunistic pathogen Pseudomonas fuscovaginae contains a conserved quorum sensing system involved in virulence
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 2600)
AUTHORS Venturi,V.
TITLE Direct Submission
JOURNAL Submitted (27-FEB-2008) Venturi V., Bacteriology, I.C.G.E.B, Padriciano 99, 34012 Trieste, ITALY
FEATURES Location/Qualifiers
source 1..2600
/organism="*Pseudomonas fuscovaginae*"
/mol_type="genomic DNA"
/strain="LMG12428"
/specific_host="*Oryza sativa*"
/db_xref="taxon:50340"
/country="Colombia"
misc_feature 1..2600
/note="quorum sensing locus"
gene 289..1008
/gene="*pfvR*"
CDS 289..1008
/gene="*pfvR*"
/function="LuxR-family quorum sensing sensor/regulator"
/codon_start=1
/transl_table=11
/product="pfvR protein"
/protein_id="CAQ15951.1"
/db_xref="GI:182375406"
/translation="MLLMDECIELLSAMDARTWFQSLVLSVQKLGYSQ
VLYALKPNKEAQNSTARIMSNFPDAWRDLYDNENYSSIDPVVSHSFSS
SLPILWEDKLYKTRREKEFAEDARRAGLSHGLTLPIHGPQGQVGMLSL
SCAPMPQNEYLQVLQHSIAAASMLRDYAVTSGSSLFLHNSSSTAPHLT
PRELEILHWAWAEKTTWEIGKILSLAEPTVEFHFKNIRRKLNVSSRRL
AVARAIQLDLMTP"
gene complement(988..1233)
/gene="*rsaL*"

```
CDS             complement(988..1233)
                /gene="rsaL"
                /function="transcriptional repressor of the
                  quorum sensing system"
                /codon_start=1
                /transl_table=11
                /product="RsaL protein"
                /protein_id="CAQ15952.1"
                /db_xref="GI:182375407"
                /translation="MRVERSLIRQAVVLRLQLGKSQEAFWRMFGLSQP
                AASRLECAGKGSASLAVLLGLYVAGRIDDDDLLFVSAREVALGRHEI"
gene            1415..1960
                /gene="pfvI"
CDS             1415..1960
                /gene="pfvI"
                /function="N-acyl homoserine lactone synthase"
                /codon_start=1
                /transl_table=11
                /product="pfvI protein"
                /protein_id="CAQ15953.1"
                /db_xref="GI:182375408"
                /translation="MHILRAKIFRDKKDWDVSVIGGMEIDGYDALNPY
                YMILQDADLNESVRGCWRLLPTTGPNMLADTFPELLAGAPVPRAEDTW
                ELSRFAICQQSGRPYAFSDQSLIAIRAVVHFGVERGLKRFVTVTTVGV
                EKLLIRLGLDIRRLGPAKTIGVERAVALSIALNHKTLDALETTGAASN
                LSN"
```

ORIGIN

```
       1 ccgtcggaca gctgaccaac gagtaactgc gtaccagccc gttgggcaga tgcagatcaa
      61 tatgggctcc agcctcgaat gccggtaagc gatcggagcc gaccggagcc aggtctatcg
     121 agacgatact gggggtttcc cggatgatgt tcctgacgcg gacggtctga aggctagcgt
     181 tcataactac ttccttgtat atatccaaga atagaggcta ttgacctcgt ttggagcagg
     241 cccctatcgc tgacaaaaat aacgattcac tgataccgga ttttttctat gctacttatg
     301 gatgaatgta tagagctgct aagtgcaatg gacgcacgaa cctggttcca gtcacttgtt
     361 ttgtccgtgc agaaactcgg atacagccag gtgctgtatg ccctgaaacc caacaaagag
     421 gcacagaaca gcacggcacg aatcatgagc aactttccgg acgcctggcg agatctttat
     481 gataacgaga actactccag catcgaccct gtcgtctccc acagcttttc atcctcgctt
     541 cccattctct gggaggacaa gctctacaaa acccgtcgag aaaaggagtt tgccgaggat
     601 gcccgacgag ccggactcag ccacggcctg acactgccca ttcacgggcc ccagggccag
     661 gtcggcatgc tcagcctgag ctgcgccccc atgccgcaaa acgagtactt gcaggtcctg
     721 caacacagca tcgccgcagc cagcatgctg cgcgactatg cggtcacgag cggcagcagc
     781 ctcttcctgc acaattccag ctcgactgca cctcacctca caccccggga actggaaatc
     841 ctgcattggg cctgggccga aaaaaccacc tgggaaatcg gcaagatcct cagtctggcg
     901 gaacccaccg tcgagttcca cttcaagaac attcgtcgca aactcaatgt cagctccagg
     961 cgactcgccg tggccagggc catacaatta gatctcatga cgccctagcg caacttcacg
    1021 ggccgacacg aagagcagat cgtcgtcatc gatccggccg gcgacataca ggccgagcag
    1081 cacggccagg ctggccgagc ccttgcctgc gcattccagc cggctcgcgg ccggctggct
    1141 caacccgaac atccgccaga acgcctcctg gctcttgccc aactgcaatc gcaggacaac
    1201 ggcctgcctg atcaaacttc gctcaaccct catttcaacg ccttcgcagg gaaacccaga
    1261 cgatattcga ctcccctccg gccagccacc tatcagattg ataggctact aattatccat
    1321 actggatata taaatacctg gtcttttcga ggtaacttcc gtgaatatag ccattgataa
    1381 aagaaacaac ttcgacgata tatgcctgga aaaaatgcat atcctgagag cgaagatatt
    1441 ccgtgataag aaagattggg atgtcagtgt catcggtggc atggagatcg atggctacga
    1501 tgcactgaat ccctattaca tgatcctgca ggacgccgat ctgaatgaaa gtgtccgcgg
    1561 ctgctggcga ctcttgccca ccaccggccc gaacatgctg gccgatactt ttccggaact
    1621 tctggcaggt gctccggttc cccgcgcgga ggacacctgg gaactcagtc gctttgccat
    1681 ctgccagcag agcggtcgcc cctatgcgtt ttccgatcaa tcgctgatcg ccatccgcgc
    1741 ggttgtccac tttggcgtcg agcgtggcct gaagcgattc gtcacggtga ccacggtggg
    1801 cgtcgagaaa ctgctcatca ggctcggcct ggatatccgg cgactgggcc cagccaagac
    1861 catcggtgtc gagcgcgccg tcgcgctgtc catcgccctg aaccacaaga cgctggatgc
    1921 cctggagaca acgggcgcgg cgtccaacct gtcaaactga taggttgtcc cgcccacgga
    1981 ttgatagatg atttggcccc acaccgggca cgccccggtt gccgctacga gacacagcta
    2041 tcaccgctct catcataaat acaattggat tgtggagacg acacatggaa accgtgactg
    2101 atttgccaaa aggcgacacc accgcattcg ccagccggga aacctggcca gccttcatca
    2161 ccgccaccgc tcagcaagac tatctgacag ccgaggcggg cctgcagcgg gccctggagc
    2221 aggggtacag ccattggtac atcgacggca gcctcgaagg cgaacgtccc tccgacttca
    2281 ccacacaacg cctggcggcg ctgaacgaac tgatcgccag cagcggcgtg aaaccgattt
    2341 tccacggcaa cttcaaggcc cccctgggca gcgatgtcga ggatctggcc gctgccgccc
    2401 tggactacgt gaaaaaagag gtcgacatct gtgccgccct gggcggtgcg ccgctgattg
    2461 tccacggagg tggcgtggtc gagccgcgcc tggtcaagga agcccgccag aagggcctgg
    2521 aacgcctggt gggcaacctg cgtgagctgg tggcctatgg caaggcccgt ggcgtcgaga
    2581 tctggctgga gaacctgtgc
//
```

2. The *Pseudomonas fuscovagine* UPB0736 quorum sensing locus (Chapter 4):

```
LOCUS       AM943857          2600 bp  DNA   linear   BCT 09-APR-2008
DEFINITION  Pseudomonas fuscovaginae quorum sensing locus,
            strain UPB0736.
ACCESSION   AM943857
VERSION     AM943857.1  GI:182375401
KEYWORDS    .
SOURCE      Pseudomonas fuscovaginae
 ORGANISM   Pseudomonas fuscovaginae
            Bacteria; Proteobacteria; Gammaproteobacteria;
            Pseudomonadales;
            Pseudomonadaceae; Pseudomonas.
REFERENCE   1
  AUTHORS   Ferluga,S., Cabrio,L., Mattiuzzo,M., Maraite,H. and
            Venturi,V.
  TITLE     The plant opportunistic pathogen Pseudomonas fuscovaginae
            contains a conserved quorum sensing system involved in
            virulence
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 2600)
  AUTHORS   Venturi,V.
  TITLE     Direct Submission
  JOURNAL   Submitted (27-FEB-2008) Venturi V., Bacteriology,
            I.C.G.E.B, Padriciano 99, 34012 Trieste, ITALY
FEATURES             Location/Qualifiers
     source          1..2600
                     /organism="Pseudomonas fuscovaginae"
                     /mol_type="genomic DNA"
                     /strain="UPB0736"
                     /specific_host="Oryza sativa"
                     /db_xref="taxon:50340"
                     /country="Madagascar"
     misc_feature    1..2600
                     /note="quorum sensing locus"
     gene            264..980
                     /gene="pfvR"
     CDS             264..980
                     /gene="pfvR"
                     /function="LuxR-family quorum sensing
                      sensor/regulator"
                     /codon_start=1
                     /transl_table=11
                     /product="pfvR protein"
                     /protein_id="CAQ15951.1"
                     /db_xref="GI:182375406"
                     /translation="MTLLVMDELLRLSEMERFEDWLAHLKILTRKLGY
                     SNFLIGLKPAPTDANQQVLIYSDYPDAWRTRYDAESYAAVDPVVQHCL
                     NANRPLLWDRDNYRRPSESEFFEEAAAHGLQQGLALPLHGPRGEAGML
                     CLKPSESGPQATATMIESLPTATLLRDYAMEGMLKARIECSTPVHLTS
                     REKEVLQWSAAGKTTWEISMILSCTTSAIDFHFKNIRRKFQVSSRQMA
                     VLKAIQQKSITP"
     gene            complement(984..1214)
                     /gene="rsaL"
```

```
CDS             complement(984..1214)
                /gene="rsaL"
                /function="transcriptional repressor of the
                 quorum sensing system"
                /codon_start=1
                /transl_table=11
                /product="RsaL protein"
                /protein_id="CAQ15952.1"
                /db_xref="GI:182375407"
                /translation="MKLLNTSAVREVAHQAISLRLGLKQSQTEFWSRF
                GISQACASRIECTSQVPAPVYILLRLYLSGRLEESDLAQRPQ "
gene            1353..1895
                /gene="pfvI"
CDS             1353..1895
                /gene="pfvI"
                /function="N-acyl homoserine lactone synthase"
                /codon_start=1
                /transl_table=11
                /product="pfvI protein"
                /protein_id="CAQ15953.1"
                /db_xref="GI:182375408"
                /translation="MLISIDRRTNIDPQALNAMHKLRARIFKVKKMWD
                IPLINDMEIDGYDALDPYYMIINRDDEAGYVCGCWRILPTTGPNMLAH
                TFPELLHGHPAPCSDAIWELSRFAIELPDGNRFGFSQGTTQAIHAIVS
                YAIGCGVKQFVTVTTVGVEKMLIRLGLDDLQGGGGKPRCVSKSLMLHC
                TR"
```

ORIGIN

```
        1 ttgacatgat tacgccagcg cgcaattaac cctcactaaa gggaacaaaa gctgggtacc
       61 tcccagaaca gggaggttgt ggcgggttgg gcaactgact agtgtctgcg accgcttgct
      121 tctcgtcggc gagcactcta tgcacgttga tttcaatgtg ccatgtaact atcttatcga
      181 cccacctgaa tgagggagtc gatattccct cgcgtgttgt ctcgccatga gaccaccgtc
      241 aaacggtgcc tacgaggaag ggaatgaccc tactggtaat ggatgagctg ctgcgactca
      301 gcgaaatgga gcgctttgag gactggctgg ctcaccttaa aatcctgacc cgcaagctgg
      361 gatactcgaa ctttctgatc ggtctcaagc ctgcgcccac tgacgccaat cagcaggtgc
      421 tgatctacag cgactaccct gatgcgtggc gcacacgtta cgacgccgag tcctacgctg
      481 cggtcgatcc ggtggttcag cactgcctga acgcaaaccg gccactgctc tgggaccggg
      541 acaactaccg ccgtccctca gagtcggaat tcttcgaaga agccgccgca catggcttgc
      601 agcaaggcct tgcgctgccc ttgcatggtc cgcgcggcga ggcgggcatg ctctgcctga
      661 aaccctctga aagtggtccg caagcgactg ctacgatgat cgagtccttg cctacggcga
      721 ccctgctgcg cgattacgcg atggagggga tgctcaaggc acgcatcgaa tgctccacac
      781 ccgtgcacct gacgagccgg gaaaaagagg tgctgcagtg gagcgcagca ggcaagacca
      841 cctgggaaat ctcgatgatt ctgtcgtgca cgacctcggc catcgacttc cacttcaaga
      901 acatccgccg caagttccag gtcagctcgc gccagatggc ggtgctcaaa gccatccagc
      961 aaaaatcgat cacgccctga gtgtcactgc gggcgttggg ccaggtcgct ttcctccagg
     1021 cgaccgctga ggtaaaggcg cagcaggata tacaccgggg cgggcacttg actggtgcat
     1081 tcgatgcggc tggcgcaggc ctggctgatg ccgaaccttg accagaattc ggtctggctc
     1141 tgcttgaggc ccaggcgcag gctgatggct tgatgggcga cctcgcgcac agctgaggta
     1201 ttgagtagct tcatcgggaa tcaatcctgc aaaagccgaa aaattaacaa gtctggttaa
     1261 tgtcggccac ctcccaaatt aggtaggatt ttatttaacc ggcatgaata taccgtgggt
     1321 ggcaattcaa acccctgccg gtaattgccc ctatgcttat ctccattgat cgccgcacta
     1381 acatcgaccc gcaagcgctg aatgccatgc ataaacttcg ggcacgtatc ttcaaagtta
     1441 aaaagatgtg ggacatcccg ctgatcaatg acatggagat cgatggctac gatgcgctcg
     1501 acccctatta catgatcatc aaccgcgatg acgaggcggg ctatgtctgt gggtgctggc
     1561 ggatactgcc gaccacgggc ccgaacatgc tggcgcatac cttccccgaa ctgctgcatg
     1621 ggcatcctgc accgtgctcc gacgccatct gggagctgag ccgctttgcc atcgagctgc
     1681 cggacggcaa tcggttcggc ttctcccagg gcaccacgca ggctattcat gcgatcgtca
     1741 gctatgccat tggctgtggc gtgaaacagt tcgtcacggt gaccacggtg ggggtcgaga
     1801 aaatgttgat ccgcctgggg ttggatgacc tgcaggggcg gggggaaag ccacgttgtg
     1861 tctcaaaatc tctgatgtta cattgcacaa gataaaaata tatcatcatg aacaataaaa
     1921 ctgtctgctt acataaacag taatacaagg ggtgttatga gccatattca acgggaaacg
     1981 tcttgctcga ggccgcgatt aaattccaac atggatgctg atttatatgg gtataaatgg
     2041 gctcgcgata atgtcgggca atcaggtgcg acaatctatc gattgtatgg gaagcccgat
     2101 gcgccagagt tgtttctgaa acatggcaaa ggtagcgttg ccaatgatgt tacagatgag
     2161 atggtcagac taaactggct gacggaattt atgcctcttc cgaccatcaa gcattttatc
     2221 cgtactcctg atgatgcatg gttactcacc actgcgatcc ccgggaaaac agcattccag
     2281 gtattagaag aatatcctga ttcaggtgaa aatattgttg atgcgctggc agtgttcctg
     2341 cgccggttgc attcgattcc tgtttgtaat tgtcctttta acagcgatcg cgtatttcgt
     2401 ctcgctcagg cgcaatcacg aatgaataac ggtttggttg atgcgagtga ttttgatgac
     2461 gagcgtaatg gctggcctgt tgaacaagtc tggaaagaaa tgcataagct tttgccattc
     2521 tcaccggatt cagtcgtcac tcatggtgat ttctcacttg ataatcttat tttgacgagg
     2581 gaaattaata cgttgtattg
//
```